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Associations between vitamin D status and blood lipid parameters in healthy, older adults

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by

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FREQUENTLY USED NOMENCLATURE

1αOHase	CYP27B1 enzyme
1,25OHD	$1,25(OH)_2D$; Calcitriol
25OHD	Calcidiol
BP	Blood Pressure
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DBP	Vitamin D Binding Protein
HDL	High-Density Lipoprotein Cholesterol
HTN	Hypertension
IOM	Institute of Medicine
LDL	Low-Density Lipoprotein Cholesterol
PTH	Parathyroid Hormone
TC	Total Cholesterol
TG	Triglyceride
VDR	Vitamin D Receptor

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ABSTRACT

Background. One explanation for the relationship between vitamin D and cardiovascular disease (CVD) mortality could be related to altered serum lipids. This study aimed to determine the association between 25OHD and serum lipid concentrations in healthy, older adults.

Methods. Serum lipid panels and 25OHD concentrations were obtained on 190 adults (median age 67 years) after a 12-hour overnight fast. 25OHD was measured by the DiaSorin Liason system. Subjects were stratified by 25OHD concentrations: <20.0 ng/mL, 20.0-29.9 ng/mL, 30.0-39.9 ng/mL and ≥40 ng/mL. One-way ANOVA was used to observe unadjusted differences and Student's *t*-tests were used to determine differences between groups. Multiple regression analyses were performed to determine the ability of 25OHD to predict serum lipid concentrations when accounting for BMI, sex, age, and statin use. Stepwise regression analyses were used to establish the best prediction model for each outcome.

Results. There was a significant relationship between 25OHD and BMI (p<0.001), glucose (p=0.042), TG (p=0.020), TC (p<0.001), LDL (p<0.001) and the TC:HDL ratio (p<0.001), but not for blood pressure, HDL, or TG:HDL ratio. When comparing subjects with 25OHD ≥40.0 ng/mL to those with <20 ng/mL, those in the highest group had a significantly lower BMI (26.1 ± 0.7 and 29.9 ± 0.8 kg/m^2), TG (100.9 ± 7.8 and 130.4 ± 9.5 mg/dL), TC (182.7 ± 5.2 and 217.4 ± 6.3 mg/dL), LDL (100.3 ± 4.5 and 131.2 ± 5.4 mg/dL), and calcium (9.67 ± 0.05 and 9.50 ± 0.06 mg/dL) concentrations, and had significantly lower TC:HDL (3.09 ± 0.15 and 4.15 ± 0.08) and TG:HDL (1.79 ± 0.22 and 2.56 ± 0.27) ratios.

When adjusting for confounders, the relationship between 25OHD and TC (p<0.001) and LDL (p<0.001) remained significant, but that between TC:HDL and TG:HDL did not. When a stepwise regression analysis was performed to determine the

best model for predicting serum lipid parameters, each 10 ng/mL increase in 25OHD predicted a statistically significant 6.72 mg/dL decline in TC (p<0.001) and 5.55 mg/dL decline in LDL (p<0.001).

Conclusion. 25OHD status is associated with favorable concentrations of TG, TC, LDL and lipid ratios in older adults. When adjusting for confounders, this relationship remains for TC and LDL. Altered lipid metabolism may help explain the association between vitamin D and CVD mortality.

CHAPTER I

INTRODUCTION: THESIS FORMATTING

This thesis will begin with a review of the pertinent literature pertaining to vitamin D and blood lipid parameters. Background will be provided regarding vitamin D metabolism, followed by an overview of classical functions of vitamin D and ending with a synopsis of vitamin D in relation to cardiovascular disease and, specifically, blood lipid parameters. After the literature review, a prepared manuscript for publication will be presented; this manuscript will meet criteria for submission the *Journal of Nutrition*. This manuscript will begin with an abstract followed by an introduction, methods section, results of the study and end with a discussion of findings.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Vitamin D is a fat-soluble vitamin that functions as a steroid hormone and can be obtained from the diet or from endogenous synthesis in the skin with exposure to ultra-violet B (UVB) light. Traditionally, we think of vitamin D for its role in calcium and phosphorus homeostasis. However, vitamin D is a prehormone with numerous extraskeletal roles, and interest in these non-traditional functions is increasingly popular in the literature. In particular, the role of vitamin D in chronic diseases is currently an area of unremitting attention; one emerging area of vitamin D research is the relevance of vitamin D status to cardiovascular disease, the number one cause of mortality in the United States (1). This literature review aims to summarize the metabolism and function of vitamin D, give an overview of its classical functions, introduce novel applications of its use and give an in-depth review of vitamin D as it pertains to cardiovascular disease and, specifically, serum lipid concentrations.

Vitamin D

Vitamin D Metabolism

Vitamin D is a steroid hormone that is unique in comparison to other vitamins in that it can be synthesized endogenously by the skin photochemically from 7-dehydrocholesterol. 7-dehydrocholesterol is a sterol produced by animals and is located in the skin, with the highest concentrations in the stratum basale and stratum spinosum. When skin is exposed to radiation in the optimum wavelengths for the production of vitamin D, between 290 and 320 nanometers, 7-dehydrocholesterol in the dermis and epidermis is converted to previtamin D (2–4). Previtamin D is subsequently converted to either vitamin D_3 (cholecalciferol) via three heat-dependent

isomerization reactions, or undergoes further photoisomerization to non-vitamin D molecules, tachysterol, lumisterol, or toxisterol. Toxic concentrations of vitamin D cannot be reached due to synthesis in the skin. Maximum vitamin D synthesis occurs in a few minutes of optimum wavelength UVB rays on light skin; at midday at an appropriate zenith angle for vitamin D synthesis, optimum levels may be reached with full body exposure in as little as one minute and the initiation of skin damage occurs after approximately 15 minutes (5). Further exposure initiates photodegradation and increases the concentration of the aforementioned non-vitamin D molecules, but does not increase vitamin D concentrations despite linear increases in DNA damage (3,4). The amount of vitamin D synthesized in the skin depends on initial 7-dehydrochoelsterol concentrations, skin pigmentation, season, time of day, latitude, sunscreen use, amount of skin exposed, and age (3,6).

Vitamin D may also be obtained from the diet naturally in a small number of foods (e.g. fatty fish), fortified into food products (e.g. dairy products), or obtained in a supplement form (7). Similarly to other fat-soluble vitamins, vitamin D is absorbed with dietary fat in the small intestine and then transported into the lymphatic system in chylomicrons. Supplemental forms of vitamin D can also be found in the plant- or fungal-derived form, vitamin D_2 (ergocalciferol), which was previously shown to be less effective at increasing serum levels of 25OHD (the storage form of vitamin D) (8–10), but this difference in efficacy has recently been challenged (11–13). From this point onward, both vitamin D_2 and vitamin D_3 will be referred to collectively as vitamin D unless otherwise specified. Regardless of its origin, vitamin D is transported via the vitamin D binding protein (DBP) to the liver, where the first hydroxylation to 25OHD (calcidiol) occurs. This reaction is catalyzed by one of several cytochrome p450 enzymes (14,15). Calcidiol enters the circulation bound primarily to DBP; it has a half-life of approximately 15 days and is the most commonly used and appropriate measurement of vitamin D status (16).

The second hydroxylation reaction occurs primarily in the kidney and converts calcidiol to the physiologically active $1,25(OH)_2D$ (calcitriol), or activated vitamin D (17). The calcidiol-DBP complex enters the kidney from the plasma through glomerular filtration and subsequent uptake occurs in the proximal tubular cells by megalin and cubilin via endocytosis (18). The enzyme CYP27B1 (1αOHase) is primarily responsible for catalyzing the second hydroxylation of 25OHD to $1,25(OH)_2D$ in the kidney. Contrary to the first hydroxylation reaction in the liver, the activity of this enzyme, thus activation of calcidiol to calcitriol, is under tight regulation by the parathyroid hormone (PTH), calcium, phosphate, calcitonin, and fibroblast growth factor 23. 1αOHase is also self-regulated by negative feedback, preventing excessive formation of the physiologically active calcitriol. Following activation, calcitriol is then transported in the circulation bound to DBP and acts on tissues displaying the vitamin D receptor (VDR). Calcitriol is a greater affinity ligand for VDR than is the calcidiol precursor. However, studies done on CYP27B1 knockout mice conclude that calcidiol also has direct gene regulatory function and acts synergistically with calcitriol (19,20). The half-life of calcitriol is 10-24 hours (4). Both calcidiol and calcitriol may be inactivated by CYP24A1, which 24-hydroxylates the molecules and subsequent catabolism deems them water-soluble, allowing for excretion in the urine (15).

Measuring Vitamin D

Measuring calcidiol is more appropriate than calcitriol for assessing vitamin D status because calcitriol has a short half-life, it does not increase directly with intake or synthesis from 7-dehydrocholesterol, and concentrations of calcitriol may be maintained even in the instance of vitamin D insufficiency. Relatively high variability in vitamin D values can be problematic when comparing concentrations across studies, so it is important to consider the method of measurement and to understand the benefits and limitations of each (21). First, it should be noted whether serum or plasma is used for reading vitamin D levels, as plasma samples read approximately 5 ng/mL higher

than serum levels (22). Different assay types include liquid chromatography/mass spectrometry, high performance liquid chromatography, radioactive immunoassay, and chemiluminesence protein-binding assays; differences in measurement may occur between assay types, especially when vitamin D_2 is supplemented as opposed to D_3 (23,24). The LIAISON 25(OH)D chemiluminescence protein-binding assay has been validated by several different research groups as an appropriate measurement method (22,23,25).

Vitamin D Intake and Status

The optimal range of vitamin D concentrations to attain the greatest benefits to health is an ongoing debate (26,27). In 2011, the Institute of Medicine (IOM) (27) and the Endocrinology Society (26) both published reports addressing the issue and, although there were similarities between the two, both made different conclusions relating to the classification of vitamin D deficiency. The IOM stated that a vitamin D concentration of 20 ng/mL is adequate for bone health for most people and they did not deem the evidence for the many non-traditional roles of vitamin D sufficient enough to support higher recommendations at the time the report was published (27). Conversely, the Endocrine Society recommended a vitamin D concentration of at least 30 ng/mL and deemed the evidence supportive of vitamin D for the prevention of falls and suppression of PTH concentrations, which are associated with increased mortality and heart failure (26,28,29). The American Dietetic Association (now called the Academy of Nutrition and Dietetics) supported the IOM guideline of a vitamin D concentration ≥20 ng/mL to provide optimum bone health (30). Some researchers who work closely with vitamin D stand behind a more liberal optimum range of 40-60 ng/mL, but this is not adopted or universally accepted by all (31,32). Evidence to support either further benefits and/or potentially negative consequences of vitamin D concentrations higher than traditionally recommended is necessary.

Vitamin D optimization enables calcium absorption but increasing concentrations of 25OHD does not cause an increase in calcium uptake until toxic levels (100-150 ng/mL) are reached; levels of calcidiol in the blood do not correspond to concentrations of the physiologically active calcitriol, and this active form can potentially be elevated due to vitamin D insufficiency. Both the IOM and the Endocrine Society stood behind an upper limit of 25OHD of 100 ng/mL for safety from vitamin D intoxication (26,27), but some vitamin D researchers trust that there are no adverse effects with calcidiol levels up to 150 ng/mL (6). To achieve 100 ng/mL, upwards of 10,000 IU/day would need to be consumed for a length of time. A general rule of thumb is that regular intake of 100 IU will increase blood levels by approximately 1 ng/mL, although the rate of rise in blood concentrations is dependent on the initial blood values as well as other characteristics and factors that can affect vitamin D metabolism (e.g. kidney function (33) and amount of adipose tissue(13)). Those deficient in vitamin D have a faster rise in calcidiol concentrations with supplementation than those with adequate levels before supplementation. Blood concentrations of vitamin D in overweight or obese individuals respond less quickly to supplementation due to sequestration in adipocytes (34,35).

There has been a lot of recent discussion about the optimal range for vitamin D status, the incidence of vitamin D deficiency, and the effects of vitamin D insufficiency. Disagreements related to optimum blood concentrations lead to arguments about appropriate dosages, as levels required for bone health are lower than those shown to have physiologically significant effects on novel discoveries of vitamin D action. The IOM reevaluated the literature pertaining to calcium and vitamin D levels and concluded that vitamin D status is not a major problem and maintained the Estimated Average Requirement (EAR) of 400 IU/day for most people one year and older and a Recommended Dietary Allowance (RDA) of 600 IU/day for most people over the age of one year, except for those >70 years old, who were given an RDA of 800 IU/day (27). An upper limit of 4,000 IU/day for healthy adults was established by both committees but

the Task Force for the Endocrine Society guideline report recommended higher levels (up to 10,000 IU/day) for adults who are deficient or at risk for deficiency (26,27).

Both the IOM and Endocrine Society acknowledged that several factors affect vitamin D status aside from dietary intake. First and foremost, the amount of UVB exposure has a large impact on vitamin D status, as synthesis from the skin can contribute to serum concentrations to a greater extent than amounts obtained from diet, especially from food alone. As expected, locations farther from the equator have a higher incidence of vitamin D deficiency, particularly in the winter months (6). In addition, vitamin D is only synthesized in the midday hours, when UVB concentrations in the appropriate wavelength are highest. Even in areas with adequate UVB radiation in the optimum wavelength, vitamin D deficiency is more prominent in the frail, bedridden, and elderly because of insufficient sun exposure and/or reduced capacity for synthesis (6,26,27).

Certain changes in metabolic function are associated with a lower vitamin D status, but whether or not certain populations should have increased levels of vitamin D intake is another point of contention (26,27,36). Most recognized is an increased need for vitamin D in the older population due to less time outdoors, less dairy consumption, and the decreased capacity to synthesize vitamin D from 7-dehydrocholesterol in the skin (6,26,37). This issue is addressed with the increased RDA value of vitamin D for those >70 years old, yet vitamin D status and intake are still lower in this population (7,27,36,38). The Endocrine Society also acknowledges a benefit of having a vitamin D concentration above 30 ng/mL for the prevention of falls in this group (26,39).

Other diseases and conditions are associated with metabolic changes that may impact or may be impacted by insufficient vitamin D. Chronic kidney diseases or impaired renal function impact the uptake and subsequent activation of calcidiol to calcitriol, thus implicating those with kidney disease or those at risk for kidney problems (e.g., diabetics) with a higher risk for insufficient calcidiol and calcitriol

concentrations (6,33). Obese individuals are at a greater risk for vitamin D deficiency, potentially due to its sequestration in the adipose tissue (35,40–42). On the other hand, adipocytes contain VDR and the ability to synthesize 1αOHase and may instead regulate adipocyte differentiation and metabolism by inhibiting or promoting adipogenesis via VDR. The underlying mechanisms for either explanation for the association between decreased vitamin D and increased adipose tissue mass have not been elucidated (42,43). Consequently, lower vitamin D status is associated with several chronic disease profiles linked with obesity, such as the metabolic syndrome, cardiovascular disease, diabetes and certain types of cancer. More detail on these associations and potential underlying mechanisms will be provided in the subsequent sections on the mechanisms of action for vitamin D and novel revelations about its use.

Classical Mechanisms of Vitamin D

Vitamin D conveys biological activity by binding to VDR, primarily in the nuclei of target cells (44). When calcitriol binds to VDR in the nucleus, it activates VDR and, together with a retinoid X receptor, binds to DNA at the Vitamin D Response Element (VDRE), working as a ligand-activated transcription factor within the promoter region of genes to activate or repress transcription of a gene by influencing the rate of transcription (45). This process is well known for the induction of calcium channel transporters, allowing for active transport of calcium in the intestinal enterocytes in the case of lower calcium intake. Additionally, calbindin-D9k and the basolateral transient receptor potential vanilloid type 6 (TRPV6) membrane calcium pump are up-regulated by vitamin D to promote transcellular transport of calcium into the intestinal epithelia. Expression of these proteins does not necessarily correlate with intestinal calcium absorption, but transcellular transport of calcium is influenced by calcitriol, suggesting that calcitriol regulates other steps in the process of calcium absorption aside from transcription of the calbindin and TRPV6 genes (46).

In short, calcitriol enables the maintenance of blood calcium concentrations within the normal range. When blood calcium concentrations decline, the parathyroid gland senses the change and induces the secretion of PTH. PTH influences CYP27B1 and thus activation of calcidiol to calcitriol in the kidney, and its presence in the small intestine increases absorption of dietary calcium. Calcitriol also acts with PTH to activate osteoclasts, promoting bone resorption and freeing calcium to maintain homeostasis. Finally, calcitriol acts to facilitate reabsorption of calcium in the kidney. The final activation of vitamin D is suppressed by the both the presence of calcitriol, which itself inactivates 1αOHase, and restored phosphate concentrations, which halts further production of PTH. Fibroblast growth factor 23, which is released from the bone in response to an increase in phosphorus and calcitriol concentrations, suppresses the activation of calcidiol in the kidney and also induces CYP24A1, an enzyme that converts calcidiol and calcitriol to water soluble forms for excretion via 24-hydroxylation (6,15,47).

This relationship between vitamin D and calcium absorption has been well described. Consequently, calcitriol is primarily known for its activity in calcium and phosphorus homeostasis, and the use of vitamin D supplementation to improve bone density is well established (48). Modern views on vitamin D implicate its association in many other disease states and conditions, but because of the tight association with vitamin D and calcium and their concurrent use in interventions, it is sometimes difficult to assess whether or not the optimization of calcium homeostasis or vitamin D itself is conveying the effects (6,47).

Novel Actions of Vitamin D

VDRs are found in tissues fairly ubiquitously throughout the body and the activating enzyme, 1αOHase, is present in several extrarenal tissues, including the brain, prostate, breast, vasculature, and immune cells, alluding to the modernly viewed role of vitamin D in paracrine and autocrine functions outside of bone health (49–52).

In addition, megalin, a protein with several functions including uptake of vitamins (including vitamin D) and their binding proteins, is present in many tissues and organs implicated in disease states associated with vitamin D deficiency (53,54) and likely provides a mechanism for targeting vitamin D to specific cells and tissues.

Fall Prevention

Closely related to skeletal health is the role of vitamin D in muscular function and fall prevention. Muscular contractions perpetuate the process and the maintenance of bone health by providing a stimulus for adaptation (55). Aside from the benefits to bone density, muscle weakness and sarcopenia are also associated with increased PTH and low vitamin D concentrations, and supplementation with vitamin D improves functional ability in frail, elderly adults (39,56). The recognition that changes in walking pattern and muscle function emerge with low vitamin D status attributed to the recommendation that older adults (>70 years old) have a higher vitamin D intake (26).

Authors conducting a meta-analysis observed a benefit of vitamin D supplementation on muscular function, size, and/or performance for 7 of 16 randomized-controlled trials, but many studies included in the analysis were not set up specifically for this purpose and presented these parameters as secondary outcomes (57). A recent study by Close and colleagues (58) supplementing young club level athletes with a placebo, 20,000 IU/week, or 40,000 IU/week of vitamin D for 12 weeks did not observe an improvement in physical function. However, only approximately half of the participants included were vitamin D deficient and the population started with good physical performance, so it would be more difficult to see an improvement in this population than with a vitamin D deficient group because of the lowered potential for gains in performance.

A meta-analysis by Bischoff-Ferrari (59) aiming to look at the efficacy of supplemental cholecalciferol on preventing fractures concluded that 700-800 IU/day, achieving concentrations of approximately 24 ng/mL, reduces the risk of fracture. The

same analysis found a reduced relative risk of falls by 19% with supplemental doses of 700-1000 IU vitamin D/day. Pfeifer and colleagues (60) supplemented 242 older adults with 500 mg calcium or 500 mg calcium plus 400 IU cholecalciferol at breakfast and dinner for one year and found significant improvements in muscular strength, improved up-and-go test time, and decreased body sway and falls in the calcium plus vitamin D group. Moreira-Pfrimer et al (61) observed an improvement in muscular strength in institutionalized elderly people after supplementation of calcium and cholecalciferol, independent of physical activity. To separate the effects of vitamin D from calcium, Gloth et al. (56) supplemented frail, elderly people who were deficient in vitamin D (<15 ng/mL) with vitamin D alone and noticed gains in functional improvement in those increasing calcidiol concentrations by at least 3 ng/mL.

Mortality

Several large, epidemiological studies and meta-analyses have found associations between low vitamin D status and an increased risk for overall premature mortality (6,36,52,62). Specifically, a recent analysis of the associations between all-cause mortality and cause-specific mortality found strong associations between vitamin D status and mortality from all causes, cardiovascular disease, cancer, and respiratory diseases in a middle-aged, German population (63). Another recent meta-analysis by Zittermann and colleagues (62) found a relative risk of 0.71 for the highest compared to the lowest quintile of vitamin D status and provided further evidence for a greater benefit with a vitamin D status in the 40-60 ng/mL range. The reasons behind a higher risk for mortality with inadequate vitamin D status haven't been completely elucidated and are multifactorial. Studies on mice without VDR can provide a lot of insight into the many metabolic disturbances related to vitamin D, but human studies with direct evidence are just beginning to appear (64).

Immunity and Inflammation

Several cell types in both the innate and adaptive immune system display VDR and can synthesize 1αOHase, alluding to a role of vitamin D in immunity (50,65,66).

Calcitriol is an immunoregulatory hormone with well documented inhibitory effects of macrophages and dendritic cells and has a beneficial role in Th1 cell mediated autoimmune disease (e.g., type I diabetes and lupus). Additionally, Boonstra et al. (67) demonstrated that calcitriol affects T helper (Th) cells by inhibiting Th1, cells overproduced in autoimmune diseases, and enhancing Th2 cell production. Disease-activated macrophages have the ability to produce calcitriol in quantities sufficient to be detected in circulation (68) and likely convey local immunoregulatory function (49).

Obesity

Vitamin D levels are inversely correlated with adiposity (34,35,40,42,69). Specifically, those with low vitamin D are at a greater risk for subcutaneous and visceral adiposity associated with metabolic syndrome. Because many studies involving vitamin D supplementation also include calcium, it is difficult to differentiate whether or not conditions related to increased adiposity are associated with vitamin D, calcium, or both. Higher intakes of calcium are inversely related to body mass, and supplementation with calcium slightly decreases adiposity (70). Additionally, fat mass should be taken into consideration when determining appropriate dosages of vitamin D, as body mass index (BMI) is inversely associated with the rate of increase in calcidiol concentrations following supplementation (34).

Intervention studies aimed at evaluating the effects of vitamin D supplementation on obesity and its metabolic effects have not fully supported a beneficial role of vitamin D on body weight (71). One study by Salehpour et al. (72) observed a decrease in fat mass after supplementing overweight and obese women with 1,000 IU vitamin D/day for 12 weeks, but did not see a change in body weight or waist circumference. Wamberg et al. (73) conducted a double-blind randomized-controlled trial (RCT) on 52 overweight, vitamin D-deficient adults and found no beneficial effect of 7,000 IU vitamin D/day for 26 weeks on insulin resistance, blood pressure, plasma lipids or inflammatory markers. A recent double-blind RCT did not observe an effect of vitamin D on weight loss, but showed a greater increase in calcidiol

concentrations in the weight loss plus vitamin D group than with either weight loss or vitamin D supplementation alone, and concluded that vitamin D supplementation may attenuate the loss of bone mineral content associated with weight loss (74).

The association between adiposity and low vitamin D should be considered when reflecting upon associations and potential cause-and-effect relationships between vitamin D and diseases associated with increased adiposity (e. g., certain types of cancer, diabetes, and cardiovascular disease).

Cancer

The genes related to calcium and phosphorus homeostasis that are controlled by vitamin D are only a few of the 200+ genes regulated by vitamin D; among these are genes related to the cell cycle. There is an association between distance from the equator and the incidence of cancer and, specifically, an inverse relationship between vitamin D concentrations and several cancer types. Tissues implicated in cancer types associated with vitamin D often display both VDR and 1αOHase, such as the breast, colon, and prostate (6). Additionally, megalin, an endocytotic receptor protein which can internalize the vitamin D-DBP complex, is expressed in some of these tissues (54). A clinical study supplementing calcium plus vitamin D showed a reduced risk for all cancers of approximately 60% (75) and vitamin D may be a protective, low-cost alternative to cancer prevention (76).

Calcitriol is known to inhibit melanoma cell proliferation and stimulates myeloid leukemia cell differentiation. It may also inhibit endometrial cancer by halting cell growth (77). Vitamin D has also proven beneficial in human skin cells, even though the sunlight associated with vitamin D synthesis has been thought to be harmful in the same effect (78,79). Vitamin D is associated with non-melanoma skin cancer, which is easily detectable and treatable, but not with melanoma, the more detrimental type of skin cancer (80). In opposition of vitamin D for the prevention of cancer risk, a recent observational study did not conclude that vitamin D was a useful measure to predict breast cancer risk and found BMI to be the only factor measured with a significant

influence (81). Definitive intervention studies and underlying mechanisms for the role of vitamin D in cancer are ongoing but inconclusive at this time.

Diabetes

Low concentrations of vitamin D are more prevalent in diabetics, and diabetics with inadequate vitamin D stores are twice as likely to develop cardiovascular disease (82). Diabetes and its associated insulin resistance and increased adiposity are strong risk factors for CVD; in addition, the effects of vitamin D on insulin secretion and insulin sensitivity may indirectly affect lipid metabolism. Calcidiol levels are inversely associated with fasting glucose levels, glucose tolerance and hemoglobin A1c (HgA1c) concentrations in adults and in children (69,83–85). In addition, 1αOHase is expressed in both adipocytes and the insulin-producing pancreatic β-cells (49,50), and insufficient concentrations of vitamin D are associated with decreased insulin sensitivity (85,86) and pancreatic beta cell dysfunction (86).

Direct supplementation with the active calcitriol showed beneficial effects on glucose clearance, glucose tolerance and insulin secretion in rats (87). A recent study by Breslavsky et al. (84), however, did not find a change in glucose homeostasis in a small group of diabetic patients supplemented with 1000 IU vitamin D/day for 12 months compared to those receiving a placebo. Further opposition to this effect in vivo was shown with a statistically significant increase in HgA1c levels after one year of vitamin D supplementation in one study, but the increase was not clinically meaningful (88).

BMI (as it relates to adiposity) and inflammatory markers are strong predictors of insulin sensitivity, and both are also associated with vitamin D, so it is difficult to differentiate between the associations and make a conclusion about the direction of causality. Additionally, diabetes is a known contributor to renal insufficiency and renal failure and, thus, is a risk factor for vitamin D deficiency due to decreased uptake and subsequent activation of vitamin D, and increased excretion in the urine (33).

Cardiovascular Disease

VDRs and vitamin D metabolizing enzymes are highly prevalent in the cardiovascular system and the strong association between vitamin D status and cardiometabolic health is likely a strong factor behind the relationship between vitamin D status and overall mortality (6,62,63,89–94). Associations between cardiovascular disease (CVD) and both season and distance from the equator give rise to the possibility of low vitamin D status being an independent risk factor for CVD. There are vitamin D-inducible genes which exert protective cardiometabolic effects, such as preventing atherosclerosis, suppressing renin and reducing myocardial damage (31,64,95). In vitro, calcitriol directly prevents foam cell production and low-density lipoprotein cholesterol (LDL) uptake by macrophages in diabetics, but the same was not observed in cells from non-diabetics (96).

Epidemiological studies provide evidence to support the notion that low levels of calcidiol are associated with CVD risk factors and can predict the occurrence of cardiovascular events, including myocardial infarction and stroke (31,89–91,97). Although the number of observational studies with significant correlations is large, some of the results and explanations for underlying mechanisms are conflicting. A recent observational study investigated the association between vitamin D status and all-cause mortality, ischemic heart disease, and stroke in 9,146 Danish individuals; the investigators found a significant association between vitamin D and all-cause mortality but this was not explained by an association between vitamin D status and ischemic heart disease, or stroke (98). Wang et al. (99) observed 1739 subjects in the Framingham Offspring Study for just over 5 years and found a graded increase in the risk for events related to CVD, with an adjusted hazard ratio of 1.62 with the lowest vitamin D concentrations (<10 ng/mL) as compared to those with a slightly higher vitamin D status (>15 ng/mL). On the contrary, a prospective study by Alele et al. (100) on 936 diabetic veterans did not show a difference in incidence of myocardial infarction, congestive heart failure or overall mortality in those in the lowest compared to those in the highest quartile of vitamin D concentrations. Looking at specific CVD risk

factors, Martins et a l(91) used the third NHANES survey to look at associations between vitamin D status and specific cardiovascular risk factors and found a greater odds ratio for the prevalence of hypertension (1.30), type II diabetes (1.98), obesity (2.29), and high serum triglyceride levels (1.47) in subjects with vitamin D concentrations in the first compared to the fourth quartile.

Randomized controlled trials confuse the conclusions about the direction of causality between vitamin D and CVD because the outcomes are diverse. While one showed modest improvements in risk factors, most did not (31,92,98,101). However, much of the intervention data reported on vitamin D status and mortality, and the risk factors, incidence and mortality from various conditions, often reported on these factors as secondary outcomes, and suffer from limitations such as poor compliance, low dosages, and populations who were not vitamin D deficient at the onset of the trial (31,101). A recent statement from the Endocrine Society recognized strong associations between vitamin D and musculoskeletal, cardiovascular, and metabolic disorders but called for additional large, long-term RCTs to clarify the preventative or treatment effects of vitamin D supplementation on these disorders (65).

Hypertension

The relationship between vitamin D deficiency and hypertension is well established and relates in part to the regulation of the renin-angiotensin system by calcitriol. This modulation occurs by way of suppressing renin gene expression via VDR, and low calcidiol concentrations may result in an up-regulation of the renin-angiotensin system (60,95,102–104). However, the differences in blood pressure between vitamin D deficient and sufficient groups is modest, at approximately 2 mmHg on average, and so improvements after vitamin D supplementation are difficult to show. Still, Pfeifer et al. (105) observed an added benefit of supplementing vitamin D plus calcium compared to calcium alone for a decreased systolic blood pressure. Additionally, exposure to UVB light and its concurrent stimulation of endogenous vitamin D production has been shown to moderate hypertension (106); the rapid production of nitric oxide (a known

modulator of blood pressure) by keratinocytes with UVB exposure may contribute to this finding (107). Despite only modest improvements in blood pressure, the role functions of vitamin D for the suppression of renin is multifactorial in its implications for atherosclerosis; high concentrations of renin are an independent risk factor for morbidity and mortality related to cardiovascular disease and are associated with the progression of thrombosis and left ventricular hypertrophy separately from its effects on blood pressure (108).

Vitamin D and Serum Lipids

Another potential contributor to the association between vitamin D and CVD could be related to serum lipid concentrations, a major independent risk factor for CVD. Association studies on vitamin D and serum lipids are plentiful, but the outcomes are mixed. Jorde et al. (109) observed an association between high vitamin D status and a favorable blood lipid profile in a cross-sectional study of 8,018 nonsmoking and 2,087 smoking subjects in Norway; those in the highest quartile of vitamin D levels had a 6.0% and 18.5% difference in high-density lipoprotein cholesterol (HDL) and triglyceride (TG) concentrations, respectively, in nonsmokers. In the 1,762 non-smoking and 397 smoking subjects in the cross-sectional, longitudinal component of the study there was an association between an increase in vitamin D over time and a decline in TG. Additionally, a recent study on 476 adults approximately 60 years of age showed a statistically significant correlation between vitamin D status and both diabetes and hypertension, but not for smoking and dyslipidemia (25).

Observed relationships between vitamin D concentrations and LDL concentrations provide diverse conclusions. In the Jorde and Grimnes review (28), five of the seven papers reporting LDL found positive associations, only one being statistically significant, while three reported negative associations, with one being significant. Karhapää et al (110) observed an inverse association between 25OHD and LDL, but found no relationship between LDL and calcitriol. Evaluating the relationship

between vitamin D and TG is also difficult; there have been both positive and negative relationships observed (28). Because both LDL and HDL may both increase with improved vitamin D concentrations, looking at the ratio of TC:HDL is more appropriate in risk determination. In the four studies in the Jorde (28) review reporting a ratio, two out of three reported statistically significant negative associations with TC:LDL, and one reported a significant negative association between vitamin D and LDL:HDL.

The associations between vitamin D concentrations and HDL appear to be the most straightforward. In a 2011 review by Jorde and Grimnes (28), all 20 publications included which reported the relationship between calcidiol concentrations and HDL observed a positive association between the two and half of these were statistically significant. However, the same research group recently observed a modest drop in HDL after vitamin D supplementation, contradicting their previous findings and evidence supporting a protective role of vitamin D on serum lipid parameters (88). Karhapää et al (110) observed an positive association between HDL and $1,25(OH)_2D$, but not for that and 25OHD.

Intervention trials do not clarify the relationship between serum lipids; supplementation of vitamin D with calcium improves lipid profiles but studies supplementing vitamin D alone are divergent, and many have found no effect (111–113). A meta-analysis by Wang et al. (114) observed a slight effect of vitamin D supplementation on LDL (3.23 mg/dL), but no effect on TC, HDL, or TG. A study by Zittermann et al. (115) displayed improvements in TG and tumor necrosis factor (TNF) with supplementation of 3,320 IU vitamin D/day compared to those receiving a placebo; the population was overweight women with low mean vitamin D levels at baseline (12 ng/mL) and the aim was to observe whether or not supplementation with vitamin D had an impact on adiposity, but no effect was found. In addition to improvements in TG and TNF, there was a worsening of LDL levels by 5.4%. A more recent study with overweight and obese women found significant improvements in HDL

(+2.7 mg/dL) but they also observed a slight worsening of both TC and LDL concentrations (116).

Furthermore, a more recent study by Jorde et al. (88) found detrimental effects on metabolic parameters after supplementing 928 middle-aged subjects with 20,000-40,000 IU/week vitamin D. Results from the intervention showed a slight decrease in HDL (3.08 mg/dL), an increase in HgA1c (0.04%) and an increase in high sensitivity C-Reactive Protein (CRP) (0.07 mg/L). However, the results are from pooled data with a variable length of supplementation and dosage. Most importantly, albeit statistically significant, the results were not clinically meaningful and are unlikely to provide evidence against supplementing high doses of vitamin D.

Cholesterol

Background

Cholesterol is a sterol molecule with crucial biological importance. It is a structural component of cell membranes, regulating fluidity and permeability, and is an essential precursor of the sex hormones and bile acids. Intermediates in the cholesterol biosynthesis pathway are also important for production of other molecules (e.g., ubiquinone), and other intermediates as well as cholesterol itself are used for posttranslational modifications of specific proteins.

Metabolism

Endogenous Cholesterol

More than half of the cholesterol in the body is synthesized endogenously in the cytoplasm of cells from acetyl CoA, with the majority being produced in the liver. Two acetyl CoA molecules condense to form acetoacetyl CoA, and another acetyl CoA molecule is added by HMG-CoA synthase to form 3-Hydroxy-3-Methylglutaryl CoA (HMG-CoA). The final step, reduction of HMG-CoA to mevalonate by HMG-CoA

reductase, is the main regulation step in the synthesis of cholesterol; the activity of this enzyme is the target of statin therapy. HMG-CoA reductase is regulated by negative feedback from mevalonate and by the final cholesterol product by way of the sterol regulatory element binding proteins (SREBP) (117).

SREBP1c is stimulated by insulin and feeding and induces enzymes in the fatty acid metabolism and TG synthesis pathway. SREBP2 is the primary responder to cholesterol concentrations in the blood and activates genes for the proteins involved in cholesterol biosynthesis and the LDL receptor (LDLR). High concentrations of cholesterol in the cell cause cholesterol to bind to proteins and prevent the cleavage of SREBPs, thus preventing the concomitant binding to sterol regulatory elements (SRE) in the nucleus, and subsequent activation of genes enhancing cholesterol synthesis and uptake (117).

Dietary Cholesterol

Cholesterol can also be obtained from animal products in the diet and this contributes approximately one third of that in the body. Dietary cholesterol is absorbed with dietary fat and fat-soluble vitamins in chylomicrons for transport to the lymphatic system and then to the general circulation at the thoracic duct.

Transport

In order to be transported in the aqueous circulation, cholesterol is carried via lipoproteins secreted by the intestine and liver. Apolipoproteins bind lipids, serve as cofactors for enzymes and are ligands for lipoprotein receptors. In addition, apolipoproteins are used to classify different types of lipoproteins in the circulation; Figure 1 shows the flow of cholesterol transport, the different lipoproteins, and their associated apolipoproteins (118).

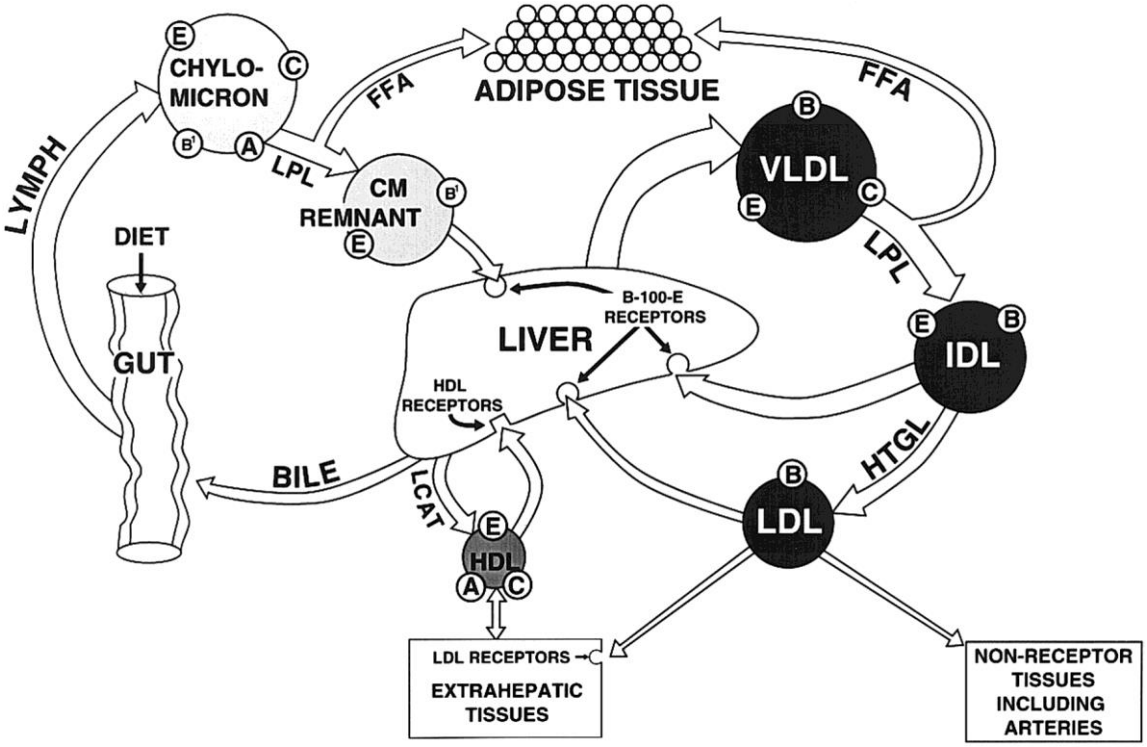

Figure 1. Overview of cholesterol transport and lipoprotein metabolism. Reprinted from Olson, 1998. J Nutr. (118)

The two primary lipoproteins are intestinal chylomicrons and hepatic very-low-density lipoproteins (VLDL). Chylomicrons are secreted from the intestine postprandially and have the largest molecular weight and lipid content. They contain apolipoprotein B-48 (apo B-48) and pick up additional apolipoproteins (C and E) from circulating LDLs to become mature chylomicrons and VLDL. Lipoprotein lipase on capillary endothelia in the circulation and hepatic triglyceride lipase (HTGL) facilitates the loss of TG from chylomicrons and VLDL to form intermediate-density lipoproteins (IDL) and chylomicron remnants; this loss coincides with the loss of apo C. Uptake of IDL by the liver is mediated by apo E. Further loss of TG (and of apo E) results in the apo B-containing LDL, which contain the highest proportion of cholesterol of all lipoproteins (118).

When considering serum lipid concentrations, LDL and HDL concentrations are considered in addition to total cholesterol, as these are the primary lipoproteins present in a fasted state. To put it simplistically, LDL cholesterol is considered detrimental to health due to its atherogenic properties, and HDL cholesterol is considered protective due to its function of returning cholesterol from the periphery back to the liver, and also due to its antioxidant properties (119). However, there are subtypes of each LDL and HDL cholesterol, which have varying degrees of risk associated with them (120). Small, dense LDL particle concentrations is a better indicator of risk than is total LDL. Similarly, small HDL particles do not yield the same protective properties as the larger HDL particles. The ratio of TC:HDL is better at predicting the amount of atherogenic subfractions than either TC or HDL alone, and is a better predictor of cardiac events (120,121). Concentrations of oxidized LDL and lipoprotein(a), a small, dense LDL particle with a second protein, apo(a), are associated with a less favorable lipid profile (119,122).

LDL Receptor Family

The LDL receptor family is a class of endocytotic transmembrane cell receptors that take up VLDL, IDL, and LDL in an apo E- and apo B-dependent manner. VLDL, IDL, LDL and chylomicron remnants are taken up by the liver via the LDL receptor (LDLR) and LDLR-related protein 1. Genetic disorders in LDL receptors are known to cause elevated LDL levels. Megalin is a newly recognized member of this family and is also the endocytotic receptor responsible for vitamin D reabsorption in the kidney (18,123).

Relationship Between Vitamin D and Cardiovascular Disease Risk Factors

Several factors may contribute to the relationship between vitamin D status and CVD risk and mortality; the incidence of obesity, amount of fat mass, adipocyte differentiation and proliferation, insulin secretion and sensitivity, blood pressure modulation, changes in blood lipids, and inhibition of atherogenesis are all associated

with vitamin D and can modulate CVD risk. Furthermore, vitamin D is inversely correlated with PTH levels, which have been linked to obesity, hypertension, heart failure, and increased mortality (28,29).

As with obesity, it is important to consider whether or not the beneficial effects of vitamin D are due to the hormone itself or its association with calcium metabolism. Calcium intake modulates the effect of dietary fat on blood lipid values, which may also explain the role of calcium on an improved cholesterol profile (124). Calcium acts to form insoluble soaps with dietary fat, preventing its absorption, thus modulating the effect of high dietary fat on blood lipid concentrations (125). In addition, calcium may also bind bile acids, the loss of which increases the hepatic conversion of cholesterol to bile acids, reducing cholesterol (126). Supporting this was a study by Reid et al (125) which supplemented calcium or a placebo to 223 post-menopausal women with a mean age of 72 years; in the intervention group, 1 g calcium was taken daily for one year and fasting serum lipid concentrations (TC, HDL, LDL and TG) were obtained at baseline and 2, 6 and 12 months. They found no effect of calcium supplementation on TG, a significant 7% increase in HDL, a non-significant 6% decrease in LDL and a significant improvement in the LDL:HDL ratio. Effects of calcium on cholesterol levels are likely more pronounced than that on TG because of the contribution of bile acid synthesis in addition to the small amount of dietary fat lost in the feces.

Vitamin D displays anti-inflammatory effects which could attribute to its beneficial effects on a variety of disease states associated with inflammation, including diabetes and cardiovascular disease. There is an inverse association between vitamin D and concentrations of CRP, a marker of inflammation and an independent risk factor for cardiovascular disease risk (29). Calcitriol acts to down-regulate pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1 and IL-8 (127,128), and up-regulate the anti-inflammatory cytokine IL-10 (129). IL-6 is produced by both T-lymphocytes and adipocytes. Beilfuss and colleagues (127) observed an

improvement in IL-6 concentrations after one year of vitamin D supplementation in obese subjects, but did not observe improved markers of insulin sensitivity.

IL-6 inhibits adiponectin, a peptide hormone with beneficial effects on atherogenesis, inflammation, glucose and fat metabolism, and obesity (127,130). Adiponectin is a cytokine released from adipocytes in relation to fat mass and is known to increase HDL levels while simultaneously having a beneficial role by lowing VLDL and LDL concentrations (131). Vitamin D levels are associated with increased adiponectin (130), and dietary interventions to improve vitamin D status coincided with an increase in adiponectin concentrations (132,133). Additionally, osteocalcin stimulates adiponectin expression and is a gene known to be up-regulated by vitamin D. The effect of vitamin D on adiponectin directly and through the down-regulation of IL-6 and/or up-regulation of osteocalcin may provide a mechanism by which vitamin D benefits CVD and CVD risk factors.

Inflammatory cytokines induce the action of endothelial 1αOHase and calcitriol modulates endothelial cell adhesion (134), modulating atherogenesis. Both endothelial cells and vascular smooth muscle cells contain VDR and calcitriol regulates many aspects of vascular smooth muscle cell function, such as contraction, growth and migration, and coronary calcification. Additionally, endothelial cells in the vasculature contain the 1αOHase enzyme, thus are able to produce active calcitriol (134). VDR deficient mice display accelerated atherosclerosis due to the effects on the renin-angiotensin system and macrophage vitamin D receptors (135). Vitamin D deficiency is associated with arterial stiffness and endothelial dysfunction (133) and supplementation with vitamin D in humans improves arterial compliance (84,133).

The mechanisms driving the association with vitamin D deficiency and both CVD risk and mortality are still being elucidated with intervention studies. Boer et al. (136) looked at vitamin D deficiency and coronary artery calcification in 1,370 participants in the Multi-Ethnic Study of Atherosclerosis and determined that lower vitamin D concentraations were not associated with coronary artery calcification, but were

associated with an increased risk for its development after adjusting for co-morbidities such as age, physical activity, CRP, season, smoking, BMI, diabetes, and serum lipids. A recent double-blind study supplemented 100 diabetic patients with 5,000 IU/day or a placebo for 12 weeks and observed no change in vascular function as evidenced by flow-mediated dilation and circulating levels of endothelial progenitor cells, nor did they see differences in high sensitivity CRP, oxidative stress markets, LDL, HDL, or HgA1C (137). Beilfuss et al. (127) observed a slight worsening in the high sensitivity CRP test in overweight and obese subjects after one year of supplementation of 20,000 or 40,000 IU/week of vitamin D.

Kidney function is strongly associated with vitamin D status, as it is the primary site for activation of calcidiol to the physiologically active calcitriol. Megalin and cubilin are necessary for filtration of the vitamin D-DBP complex in the kidney and renal insufficiency instigates vitamin D deficiency due to urinary losses and the prevention of subsequent activation (18,33). Megalin is also a member of the LDL receptor family and has a high affinity for the apolipoprotein B-containing lipoproteins. Apolipoprotein A-I and thus HDL, is a ligand for cubilin (123,138). Therefore, megalin and cubilin are important for vitamin D uptake and activation and can also facilitate the removal of lipoproteins. In turn, megalin expression is regulated by vitamin D (139).

Some researchers have observed an increase in calcidiol concentrations with statin use and this is an important consideration when making conclusions on vitamin D status and serum lipids. Effects of statin therapy on vitamin D seems to differ between statin types (140). Atorvastatin has been shown to increase calcidiol concentrations (141), but an opposite effect of Atorvastatin decreasing calcidiol concentrations has been observed (142). Until the exact relationship is determined, it is important to be aware of a potential connection between statin therapy and vitamin D concentrations.

Finally, it is important to consider whether vitamin D status is a cause or a marker for CVD. The direction of the relationship is unclear because of the association between vitamin D status and an overall healthy lifestyle. Calcidiol concentrations are

associated with being outdoors and being physically active and weight loss is associated with increased calcidiol levels (31,143).

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CHAPTER III:

ASSOCIATIONS BETWEEN VITAMIN D STATUS AND SERUM LIPID PARAMETERS IN HEALTHY, OLDER ADULTS

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Abstract

Background. One explanation for the relationship between vitamin D and cardiovascular disease (CVD) mortality could be related to altered serum lipids. This study aimed to clarify the association between 25OHD and serum lipid concentrations in healthy, older adults.

Methods. Serum lipid panels and 25OHD concentrations were obtained on 190 adults (median age 67 years) after a 12-hour overnight fast. 25OHD was measured by the DiaSorin Liaison system. Subjects were stratified by 25OHD concentrations: <20.0 ng/mL, 20.0-29.9 ng/mL, 30.0-39.9 ng/mL and ≥40 ng/mL. One-way ANOVA was used to observe unadjusted differences and Student's *t*-tests were used to determine differences between groups. Multiple regression analyses were performed to determine the ability of 25OHD to predict serum lipid concentrations when accounting for BMI, sex, age, and statin use. Stepwise regression analyses were used to establish the best prediction model for each outcome.

Results. There was a significant relationship between 25OHD and BMI (p<0.001), glucose (p=0.042), TG (p=0.020), TC (p<0.001), LDL (p<0.001) and the TC:HDL ratio (p<0.001), but not for blood pressure, HDL, or TG:HDL. When comparing subjects with

25OHD ≥40.0 ng/mL to those with <20 ng/mL, those in the former group had a significantly lower BMI (26.1 ± 0.7 and 29.9 ± 0.8 kg/m^2), TG (100.9 ± 7.8 and 130.4 ± 9.5 mg/dL), TC (182.7 ± 5.2 and 217.4 ± 6.3 mg/dL), LDL (100.3 ± 4.5 and 131.2 ± 5.4 mg/dL), higher calcium (9.67 ± 0.05 and 9.50 ± 0.06 mg/dL) concentrations, and had significantly lower TC:HDL (3.09 ± 0.15 and 4.15 ± 0.08) and TG:HDL (1.79 ± 0.22 and 2.56 ± 0.27) ratios.

When adjusting for confounders, the relationship between 25OHD and TC (p<0.001) and LDL (p<0.001) remained significant, but that between TC:HDL and TG:HDL did not. When a stepwise regression analysis was performed to determine the best model for predicting serum lipid parameters, each 1 ng/mL increase in 25OHD predicted a statistically significant 0.672 mg/dL decline in TC (p<0.001) and 0.555 mg/dL decline in LDL (p<0.001).

Conclusion. 25OHD status is associated with favorable concentrations of TG, TC, LDL and lipid ratios in older adults. When adjusting for confounders, this relationship remains for TC and LDL. Altered lipid metabolism may help explain the association between vitamin D and CVD mortality.

Introduction

Vitamin D is a fat-soluble vitamin that functions as a steroid hormone and can be obtained from the diet or synthesized endogenously with exposure to ultra-violet B (UVB) light. It is found in the diet naturally (e.g. fatty fish) artificially in fortified food products (e.g. dairy), or obtained in a supplement form. Supplements may be in the plan- or fungal-derived form, vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol). Both forms are effective at increasing serum concentrations of 25OHD, the storage form of vitamin D, and will be referred to simultaneously as vitamin D (1,2). Endogenous biosynthesis of vitamin D from 7-dehydrocholesterol occurs in the dermis and epidermis with exposure to UVB light in the optimum wavelength (between 290-

320 nanometers) and thus is dependent on season, latitude, the amount of skin exposed, skin pigmentation, sunscreen use, and age (3,4).

Vitamin D and its metabolites are transported to the liver via vitamin D binding protein (DBP), where the first hydroxylation to 25OHD, or calcidiol, occurs primarily by way of the CYP27A1 enzyme. 25OHD is the most commonly used and most effective measurement of vitamin D status (5). The second hydroxylation to the physiologically active 1,25$(OH)_2$D, or calcitriol, occurs primarily in the kidney by CYP27B1, another cytochrome P450 superfamily member (6). This activation step is reliant on the uptake of the calcidiol-VBP complex in the kidney by the endocytotic receptor proteins megalin and cubilin, for which DBP is a high-affinity ligand (7,8). 1,25$(OH)_2$D has high affinity for the vitamin D receptor (VDR) and the VDR-calcitriol complex forms a heterodimer with a retinoic X receptor (RXR). Together, they bind to the vitamin D response element (VDRE), present in the promoter region of many genes, and acts as a ligand-activated transcription factor to activate or repress gene expression, such as that for calcium channel receptors in the small intestine. CYP27B1 is under tight regulation by parathyroid hormone (PTH), calcium, phosphate, calcitonin, and fibroblast growth factor 23, in addition to negative feedback by the 1,25$(OH)_2$D end product. 1,25$(OH)_2$D concentrations are directly related to calcium and phosphorus homeostasis and are not a good indicator of vitamin D status, as concentrations are maintained or even elevated when vitamin D stores are low.

VDR is found quite ubiquitously throughout the body and extrarenal tissues express the gene for CYP27B1 as well as those for megalin and cubilin. Presence of the cellular machinery necessary for uptake and subsequent activation of vitamin D outside of the kidney alludes to a role of vitamin D in paracrine and autocrine functions (7–10). It is no surprise that several of the tissues known to express VDR, CYP27B1, megalin, and cubilin are those implicated in disease states associated with vitamin D deficiency; these include the brain, prostate, breast, vasculature, and immune cells (8–13). Consequently, several large, epidemiological studies have shown associations between

low vitamin D status and increased risk for premature mortality from all-causes, and specifically for cardiovascular disease, certain types of cancer, and respiratory illnesses (12,14,15).

There are several indirect and direct relationships between vitamin D status and cardiovascular disease and the strong associations between cardiometabolic health and vitamin D status likely contribute to the higher risk of all-cause mortality in vitamin D deficient individuals (16). Vitamin D is known to down-regulate renin, which is well-known for its implication in hypertension, but is also an independent risk factor for morbidity and mortality (17,18). Renin is associated with thrombus progression and left-ventricular hypertrophy independently of blood pressure (19). In addition, 25OHD is lower in those with obesity and diabetes, and vitamin D has known anti-inflammatory effects, all of which could modulate cardiometabolic health (15,20). PTH levels are directly affected by vitamin D status and have also been linked to obesity, hypertension, heart failure, and increased mortality (21).

There is no doubt that the relationship between cardiovascular disease and vitamin D status is multifactorial, but one potential explanation could be the affiliation of altered serum lipid concentrations with vitamin D status. Others have found associations between storage concentrations of vitamin D and serum lipids, but the outcomes have been divergent (22–25). The purpose of this study was to determine whether or not an association was present between 25OHD and dyslipidemia in an otherwise healthy group of older adults. Clarification of this relationship can aid in the appropriate target population(s) and study designs for intervention studies aimed to elucidate the relationship between dyslipidemia and vitamin D status.

Methods

Subjects

Subjects were males and females at least 60 years of age recruited from the Ames, Iowa area to be screened for an intervention study designed to look at nutritional interventions for age-related muscular function and strength loss (see Appendix A for recruitment letter). Informed consent was obtained (see Appendix B) after an explanation of what the screening appointment entailed as well as details of the study if the respondent were to qualify. Subjects were free of liver and kidney disease and of chronic disease that affects calcium or bone metabolism, not classified as morbidly obese, and free of osteoporosis or other serious medical illnesses. Participants were excluded if they had uncontrolled hypertension, were insulin-dependent, had surgery within the last six weeks, or were taking high doses of vitamin D.

Measurements

Anthropometric data (height, weight, blood pressure, and heart rate) and a blood sample by venipuncture were obtained after a 12-hour overnight fast. Fasting was verified by the phlebotomist and if a 12-hour fast was not confirmed, measurements of triglyceride and glucose concentrations were not considered. Height, weight, blood pressure and heart rate were measured in duplicate and means were reported. Blood pressure was measured after subjects were sitting for several minutes with five minutes between the two measurements. Medical history information, demographic forms, a list of medications used, the Clinical Research Health Form, the Clinical Research Feeling Form, and the SF-36 Health Survey were completed.

Serum 25OHD were measured in duplicate using the DiaSorin Liaison system, with intra-assay coefficient of variance 5.4% (Heartland Assays, Ames, IA), and the average is reported. This assay is a direct competitive chemiluminescence

immunoassay for quantitative determination of 25OHD concentrations in serum, which has been validated as an accurate and useful tool for determining vitamin D status (26,27). Blood chemical panels, which included determination of serum total cholesterol, high-density lipoprotein cholesterol concentrations (HDL), triglyceride concentrations (TG), and plasma glucose concentrations, as determined by enzymatic methods, were conducted by Laboratory Corporation of America® (Cranford, NJ). Low-density lipoprotein cholesterol concentrations (LDL) were calculated using the Friedewald equation: LDL = TC – HDL – TG*(0.2).

Statistical Analysis

First, subjects were stratified into four groups by 25OHD concentrations: <20.0 ng/mL, 20.0-29.9 ng/mL, 30.0-39.9 ng/mL, and ≥40 ng/mL. Means ± standard errors for all variables were calculated for each group and one-way analysis of variance (ANOVA) and Student's *t*-tests were used to identify significant differences between groups. Results from ANOVA were used to identify confounding variables, which were accounted for in the regression model.

Second, multiple regression analyses were performed to determine the ability of 25OHD concentration as a continuous variable to predict TC, HDL and TG concentrations and the TC:HDL ratio. Models were constructed to determine the contribution of vitamin D (constant independent variable) for predicting serum lipid parameters (dependent variable) after accounting for covariates. The first model used a multiple regression analysis for each lipid parameter while accounting for BMI, sex and statin use. The second model used a forward stepwise regression approach to determine the best prediction model using available data; variables were included in the model if the estimate for prediction was significant at $p < 0.25$. Histograms of variable distributions and residual plots of outcome variables were visually analyzed for appropriateness of models. Outcome variables that were not normally distributed were transformed for regression analyses.

Analyses were performed using JMP® 10 for Windows and significance was set at $p < 0.05$.

Results

Between January 2012 and April 2013, 190 subjects were screened in Ames, Iowa (latitude 42° N). Females comprised 83/190 (43.7%), 187/190 (98.4%) were Caucasian, mean BMI was 28.3 ± 0.3 kg/m^2, and 66/190 (34.7%) were prescribed statin therapy. Age of subjects ranged from 60-89 years with median age of 67. There were no differences between men and women for any variable measured except systolic blood pressure (BP), which was significantly lower in women (132 ± 2 mmHg) than in men (138 ± 2 mmHg).

Subject characteristics are reported in relation to serum 25OHD concentration ranges (<20, 20-29.9, 30-39.9, and ≥40 ng/mL) in Table 1. The number of subjects in each range of 25OHD levels, <20, 20-29.9, 30-39.9, and ≥40 ng/mL, was *N*=31, 59, 55 and 45, respectively. The number of subjects who were fasted at the time of the blood draw and, consequently, were included in the analysis of TG and glucose in addition to serum cholesterol concentrations, was 31, 58, 55 and 43 for each range of 25OHD concentration: <20, 20-29.9, 30-39.9, and ≥40 ng/mL. TG concentrations were normal log transformed to fit a normal distribution before regression analyses.

There was a statistically significant relationship between 25OHD and sex (p=0.024), age (p=0.006), BMI (p<0.001), glucose (p=0.042), TG (p=0.020), TC (p<0.001), LDL (p<0.001) and the TC:HDL ratio (p<0.001), but not for calcium, systolic BP, diastolic BP, HDL, TG:HDL, nor in proportions using statins. When comparing subjects with the highest (≥40.0 ng/mL) to the lowest (<20 ng/mL) concentrations of 25OHD, those in the highest range were older (69.7 ± 1.1 and 65.9 ± 1.3 years old), had a lower BMI (26.1 ± 0.7 and 29.9 ± 0.8 kg/m^2), TG (100.9 ± 7.8 and 130.4 ± 9.5 mg/dL), TC (182.7 ± 5.2 and 217.4 ± 6.3 mg/dL), LDL (100.3 ± 4.5 and 131.2 ± 5.4 mg/dL), and higher calcium (9.67 ± 0.05 and 9.50 ± 0.06 mg/dL) concentrations, and had lower TC:HDL (3.09 ± 0.15 and 4.15 ± 0.08) and TG:HDL (1.79 ± 0.22 and 2.56 ± 0.27) ratios.

There was not a significant difference between those with the highest (≥40 ng/mL) and the lowest (<20 ng/mL) concentrations of 25OHD for HDL, diastolic BP, systolic BP, and plasma glucose. Additionally, there was no difference between groups for the number of individuals with elevated creatinine levels (classified as >1.0 mg/dL for women, >1.2 mg/dL for men), or the ratio of blood urea nitrogen to creatinine (BUN:Cr), which may suggest renal dysfunction (data not shown).

After accounting for BMI, sex, age and statin use, every one-unit ng/mL increase in 25OHD was associated with an average 0.673 mg/dL decline in TC (p<0.001), and an average 0.521 mg/dL decline in LDL (p=0.003) (Table 2). There was a non-significant trend toward a lower TC:HDL ratio with a higher 25OHD (p=0.054). 25OHD status was not a statistically significant predictor of TG, TC:HDL nor TG:HDL, adjusting for BMI, sex, and statin use. When a stepwise regression analysis was performed to determine the best model for predicting serum lipid parameters, a 1 ng/mL increase in 25OHD was associated with a statistically significant mean 0.672 mg/dL decline in TC (p<0.001) and 0.555 mg/dL decline in LDL (p<0.001) (Table 3). 25OHD was not a statistically significant predictor for TG, HDL, TC:HDL or TG:HDL, as evidenced by its exclusion from the prediction model that was deemed most appropriate by a stepwise regression analysis.

Significant predictors for serum lipid parameters as determined by stepwise regression analysis (with the regression coefficient for the model) were as follows: systolic BP, BMI, calcium, glucose, and age for TG (R^2= 0.238), 25OHD, sex, diastolic BP, calcium, and statin use for TC (R^2= 0.341), sex, BMI, and glucose for HDL (R^2= 0.266), vitamin D, sex, diastolic BP, calcium, and statin use for LDL (R^2= 0.293), glucose, diastolic BP, sex, BMI, and statin use for TC:HDL (R^2= 0.181), and sex, glucose, calcium, and BMI for TG:HDL (R^2= 0.253).

Discussion

This study aimed to determine whether or not vitamin D status was associated with serum lipid concentrations in a healthy older adult population. Our data showed a

significant association between vitamin D status and a favorable lipid profile, with a decrease in TG, TC, LDL, and TC:HDL ratio with increasing levels of vitamin D. We also observed a significant association between 25OHD concentrations and differences in gender, BMI, calcium, and glucose concentrations. Because several of the variables considered are interrelated with one another in addition to being associated with vitamin D, we used regression analyses to account for potential covariates. After adjustment for covariates using a pre-determined model for all outcome variables and also using a stepwise regression analysis to determine the best prediction model, we determined that 25OHD could effectively predict serum concentrations for TC and LDL cholesterol.

Ponda et al's (24) analysis of vitamin D levels and serum lipids in a large clinical laboratory database found modest yet favorable differences between 25OHD levels and TC, LDL, HDL, and TG. Jorde et al (22) found significant and favorable differences between HDL and TG for those in the highest quartile of vitamin D status. A review by the same group concluded that HDL and vitamin D had the most straightforward relationship; 20 included publications reported a positive association between vitamin D levels and HDL, with half of these relationships being statistically significant (28). We observed a trend of higher HDL in those with better vitamin D status but the small sample size did not allow for the power to see potentially statistically significant differences in this parameter.

Results on associations between 25OHD and LDL are divergent. Jorde et al. (22) observed a detrimental, positive association between vitamin D status and the atherogenic LDL cholesterol. In contrast, Karhapää et al. (29) observed an inverse relationship between the two variables in an all-male population of a similar age. Our data support the prevalence of a beneficial inverse association between 25OHD and LDL concentrations. In addition to finding statistically significant differences between those with the highest compared to the lowest concentrations of 25OHD, we found clinically significant differences large enough to modulate cardiovascular disease risk.

Table 4 displays the optimum ranges for serum lipids as defined by the National Cholesterol Education Program (30). Those with vitamin D concentrations ≥40 ng/mL had an average TC in the "Desirable" range, as compared to a mean TC level 19% higher and classified as "Borderline High" in those with the lowest 25OHD concentrations. Similarly for LDL cholesterol, having a vitamin D level ≥40 ng/mL was associated with a more favorable interpretation of LDL values.

Interpreting the ratio of TC:HDL and TG:HDL is more enlightening than each parameter alone when determining cardiometabolic risk. TC >200 mg/dL is a risk factor for cardiac events, but if a high proportion is made up of the protective HDL cholesterol risk is lower than if the elevation is due primarily to increased amounts of the atherogenic LDL cholesterol (30). Further, subtypes of the lipoproteins are better at clarifying the risk of atherogenesis, as the smaller lipoprotein LDL and larger HDL subfractions are thought to be more atherogenic (31). Nuclear magnetic resonance (NMR) spectroscopy is a method used to differentiate between the lipoprotein particles, but it is time-consuming and expensive. The ratio of TC:HDL is better at predicting the amount of atherogenic subfractions than either TC or HDL alone, and is a better predictor of cardiac events (31,32). We observed a better TC:HDL ratio in those with higher vitamin D levels, but this relationship was lost after accounting for covariates.

The ratio of TG:HDL is another useful tool for identifying CVD risk, and a higher TG:HDL is associated with the concentration of the small, dense, atherogenic LDL particles (33); this is due to the presence of a large amount of TG-rich VLDL, which generate small, dense LDL particles after lipolysis. A high TG:HDL is an indicator of altered glucose and lipid metabolism and is associated with insulin resistance and beta cell dysfunction (33–35). As a consequence, diabetics are at an increased risk of high TG:HDL and are known to have lower vitamin D stores [33, 34] . Similarly, vitamin D is known to have a beneficial effect on insulin sensitivity and pancreatic beta cell function (36). We observed a higher fasting glucose concentration and a less favorable TG:HDL

ratio in those with higher vitamin D concentrations, but neither relationship was maintained after accounting for other variables.

One study showed that the relationship between TG:HDL and cardiac events was nonsignificant when accounting for kidney dysfunction in Type II diabetics, alluding to kidney health as an important mediator in this association (37). Kidney function is also strongly associated with vitamin D status, as it is the primary site for activation of calcidiol to the physiologically active calcitriol. Megalin and cubilin are necessary for filtration of the vitamin D-DBP complex in the kidney and renal insufficiency instigates vitamin D deficiency due to urinary losses and the prevention of subsequent activation (7,38). Megalin is also a member of the LDL receptor family and has a high affinity for the apolipoprotein B-containing lipoproteins. Apolipoprotein A-I, thus HDL, is a ligand for cubilin.(8,39) Therefore, megalin and cubilin are important for vitamin D uptake and activation and have the ability to remove lipoproteins from the circulation. In turn, megalin expression is regulated by vitamin D (40). This relationship may provide insight to a potential mechanism contributing to the relationship between vitamin D and serum lipid concentrations.

Another potential mechanism underlying changes in lipid profile and vitamin D status is that of vitamin D, serum lipids and adiponectin. Adiponectin is a cytokine released from adipocytes in relation to fat mass and is known to increase HDL levels while simultaneously having a beneficial role by lowing VLDL and LDL concentrations (41). Vitamin D levels are associated with increased adiponectin (42), and dietary interventions designed to improve vitamin D status coincided with an increase in adiponectin concentrations (43). Vitamin D status also has a negative association with arterial compliance and endothelial dysfunction (44). Supplementation with vitamin D improved Central Aortic Augmentation Index scores in addition to adiponectin concentrations independently of glucose control in diabetics (45).

Our study was limited by having a small convenience sample and the inability to make conclusions about cause-and-effect relationships between vitamin D status and

serum lipids due to a correlational approach. Additionally, this study was done as a secondary analysis of screening subjects for a randomized-controlled trial to look at nutritional interventions for the prevention of sarcopenia, so it was not designed specifically to look at this relationship. Additionally, we were unable to account for potential endogenous vitamin D synthesis. Future research should be conducted to determine whether or not supplementation with vitamin D has an impact on serum lipid parameters and to try to elucidate the mechanisms behind this relationship.

Table 1. Age, gender, BMI, blood pressure, glucose, serum lipids, and statin use in relation to serum vitamin D concentration levels in all subjects.

	Serum 25OHD Concentrations[1]				
	<20 ng/mL	20.0-29.9 ng/mL	30.0-39.9 ng/mL	≥40.0 ng/mL	P-value[2]
	N=31	N=59	N=55	N=45	
Sex (% female)	38.7	30.5	58.2	46.7	0.024*
Age (years)	65.9[a] ± 1.3	68.2[a,b] ± 0.9	68.9[a,b] ± 1.0	69.7[b] ± 1.1	0.006*
BMI (kg/m^2)	29.9[a] ± 0.8	29.4[a] ± 0.6	28.2[a] ± 0.6	26.1[b] ± 0.7	<0.001*
Statins (% using)	22.6	33.9	36.4	42.2	0.360
Calcium (mg/dL)	9.50[a] ± 0.06	9.58[a,b] ± 0.04	9.56[a,b] ± 0.05	9.67[b] ± 0.05	0.143
Systolic BP (mmHg)	133.4 ± 3.2	135.9 ± 2.3	136.5 ± 2.4	135.2 ± 2.7	0.559
Diastolic BP (mmHg)	74.2 ± 1.9	77.6 ± 1.4	74.0 ± 1.4	74.5 ± 1.6	0.342
Glucose (mg/dL)[3]	95.1 ± 2.4	97.3 ± 1.7	93.7 ± 1.8	92.7 ± 2.0	0.042*
TG (mg/dL)[3]	130.4[a] ± 9.5	119.2[a,b] ± 6.9	115.5[a,b] ± 7.2	100.9[b] ± 7.8	0.020*
TC (mg/dL)	217.4[a] ± 6.3	192.4[b,c] ± 4.6	197.8[b] ± 4.7	182.7[c] ± 5.2	<0.001*
HDL (mg/dL)	57.2 ± 3.2	58.4 ± 2.3	64.0 ± 2.4	62.1 ± 2.6	0.243
LDL (mg/dL)	131.2[a] ± 5.4	109.7[b] ± 3.9	110.4[b] ± 4.04	100.3[b] ± 4.5	<0.001*
TC:HDL ratio	4.15[a] ± 0.18	3.45[b] ± 0.13	3.33[b] ± 0.13	3.09[b] ± 0.15	<0.001*
TG:HDL ratio	2.56[a] ± 0.27	2.26[a,b] ± 0.19	2.11[a,b] ± 0.20	1.79[b] ± 0.22	0.052

Abbreviations: BMI, body mass index; BP, blood pressure; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

[1]Values listed are mean ± standard error for each concentration of 25OHD. Student's *t*-tests were used to compare means for quantitative variables; values with different letters are statistically different from one another.

[2]One-way analysis of variance to determine associations for quantitative variables and the Pearson chi-square test was used to test differences between group proportions for categorical variables.

[3]Subjects who had not fasted when the blood sample was obtained were excluded from the analysis of glucose and TG concentrations. *N* for TG and glucose in each level of 25OHD were 31, 58, 55, and 43.

*Indicates statistical significance at $p < 0.05$

Table 2. Results of multiple regression analyses to determine the ability of vitamin D status to predict serum lipid concentrations when adjusting for BMI, sex and statin use.

	β[1] of vitamin D ± Std Error	*p* for 25OHD	R^2 of model
TG (mg/dL)	-0.418 ± 0.310	0.180	0.132
TC (mg/dL)	-0.673 ± 0.194	<0.001*	0.310
HDL-C (mg/dL)	-0.024 ± 0.098	0.809*	0.256
LDL-C (mg/dL)	-0.521 ± 0.171	0.003*	0.272
TC:HDL ratio	-0.012 ± 0.006	0.054	0.179
TG:HDL ratio	-0.008 ± 0.009	0.324	0.172

Abbreviations: BMI, body mass index; BP, blood pressure; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

[1]Change in parameter per 1 ng/mL increase of 25OHD; adjusted for sex, BMI and statin use.

*Indicates statistical significance at $p < 0.05$

Table 3. Results of a stepwise regression analysis to determine the ability of vitamin D status to predict serum lipid concentrations within the best prediction model.

	β[1] of vitamin D ± Std Error	*p* for 25OHD	R^2 of model
TG[3] (mg/dL)	-0.420 ± 0.293	0.154	0.238
TC[4] (mg/dL)	-0.672 ± 0.180	<0.001*	0.341
HDL-C[5] (mg/dL)	-0.056 ± 0.096	0.559	0.266
LDL-C[6] (mg/dL)	-0.555 ± 0.159	<0.001*	0.293
TC:HDL[7] ratio	-0.010 ± 0.006	0.104	0.181
TG:HDL[8] ratio	-0.004 ± 0.008	0.576	0.253

Abbreviations: BMI, body mass index; BP, blood pressure; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

[1]Change in parameter per 1 ng/mL increase of 25OHD; best prediction model as determined by inclusion of variables with a p-value threshold <0.25.

[3]Adjusted for systolic BP, BMI, calcium and glucose and age

[4]Adjusted for sex, diastolic BP, calcium, and statins

[5]Adjusted for sex, BMI, and glucose

[6]Adjusted for sex, diastolic BP and statin use

[7]Adjusted for glucose, diastolic BP, sex, BMI and statin use

[8]Adjusted for sex, glucose, calcium, and BMI

*Indicates statistical significance at $p < 0.05$

Table 4. Optimum ranges for blood lipid parameters according to the National Cholesterol Education Program [30].

	Interpretation of Values
TG (mg/dL)	
<150	Normal
150-199	Borderline High
200-499	High
≥500	Very High
TC (mg/dL)	
<200	Desirable
200-239	Borderline High
≥240	High
HDL-C (mg/dL)	
<40	Risk Factor for CVD
40-60	Normal
≥60	Protective
LDL-C (mg/dL)	
<100	Optimal
100-129[1]	Near Optimal
130-159	Borderline High
160-189	High
≥190	Very High

[1]Not considered hazardous unless other CVD risk factors are present

Figure 1. Mean triglyceride levels across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.

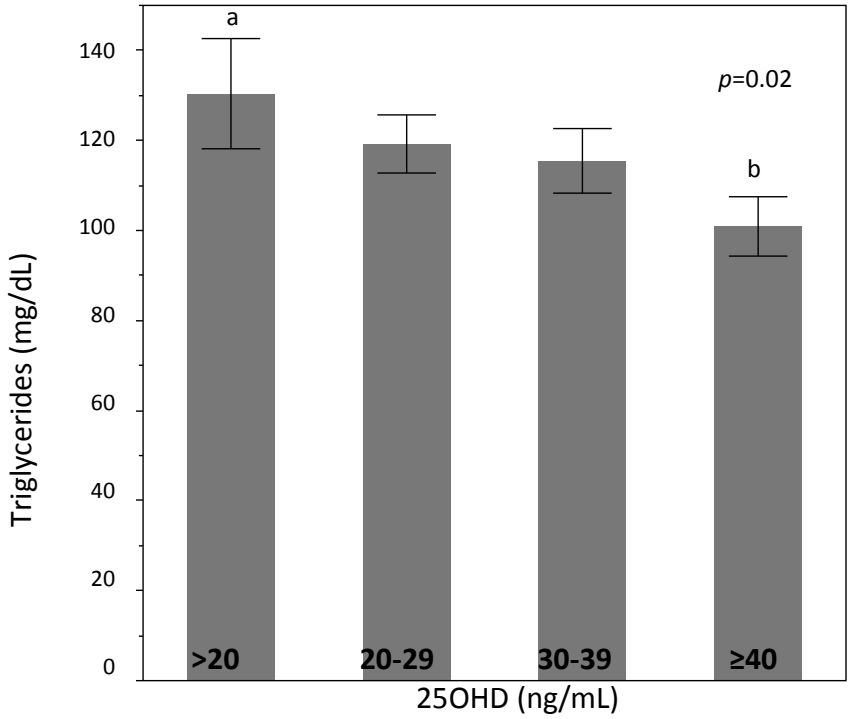


Figure 2. Mean Total Cholesterol levels across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.

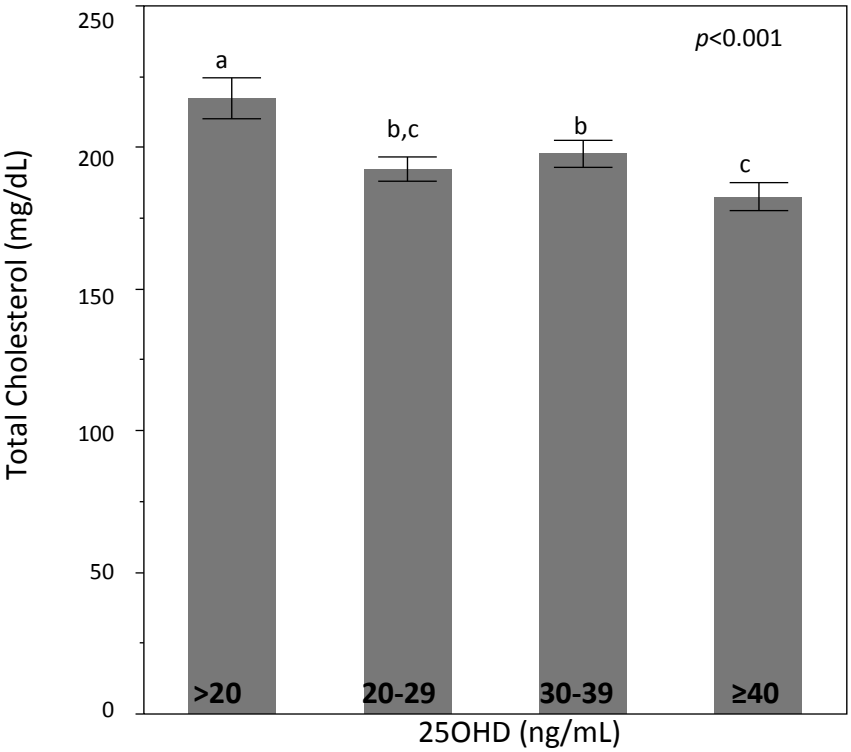


Figure 3. Mean HDL Cholesterol levels across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.

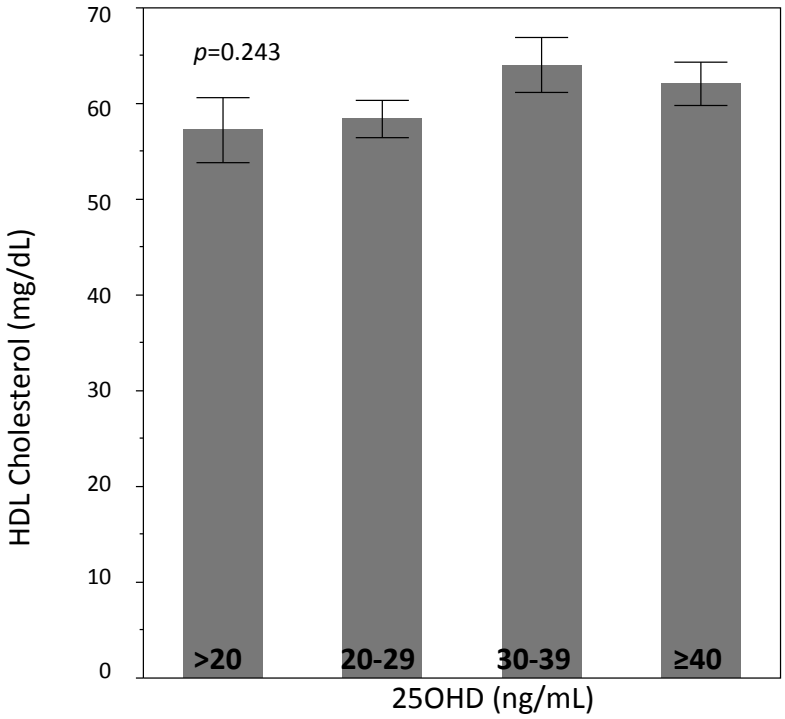


Figure 4. Mean LDL Cholesterol levels across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.

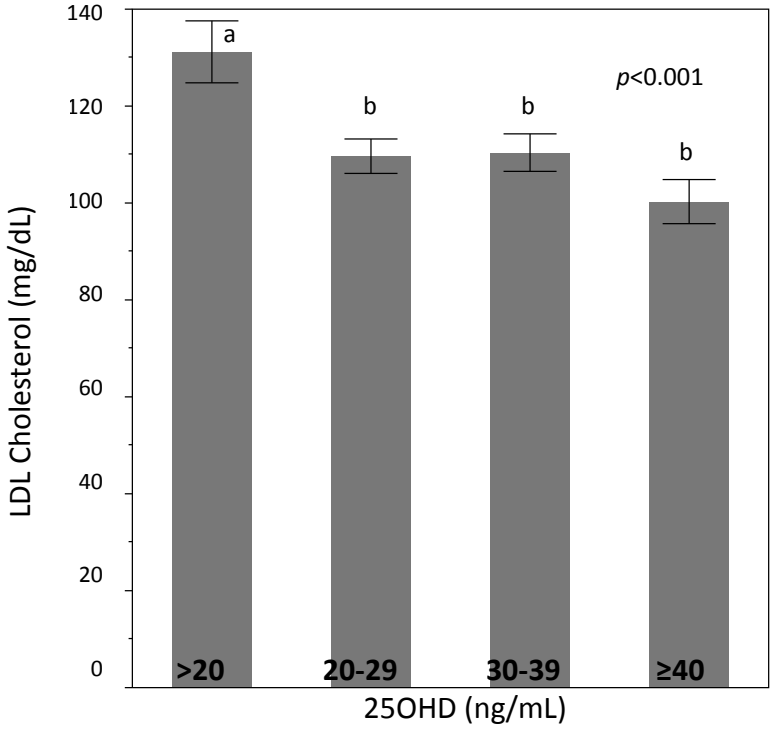


Figure 5. Mean TG:HDL ratio across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.

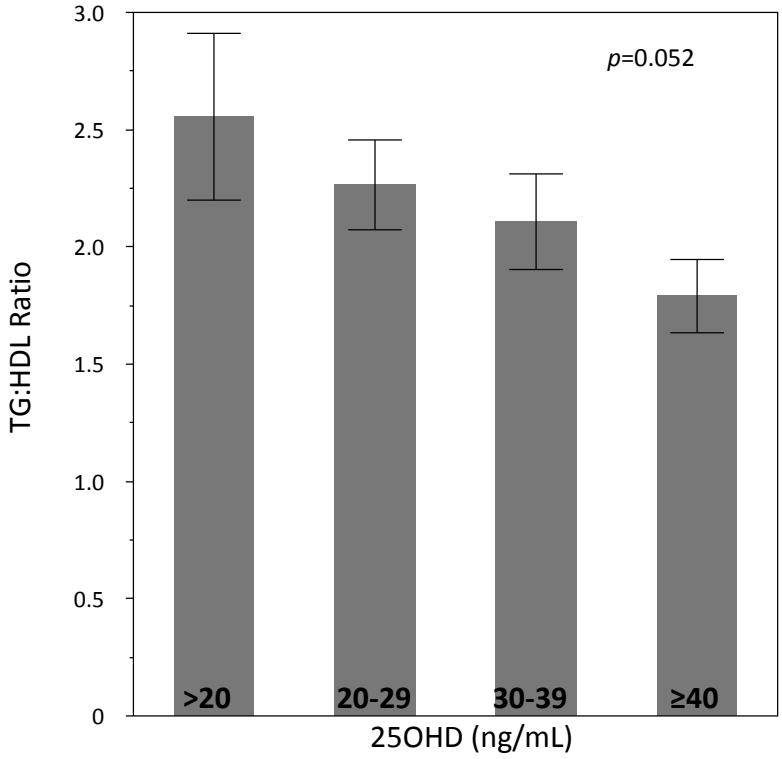
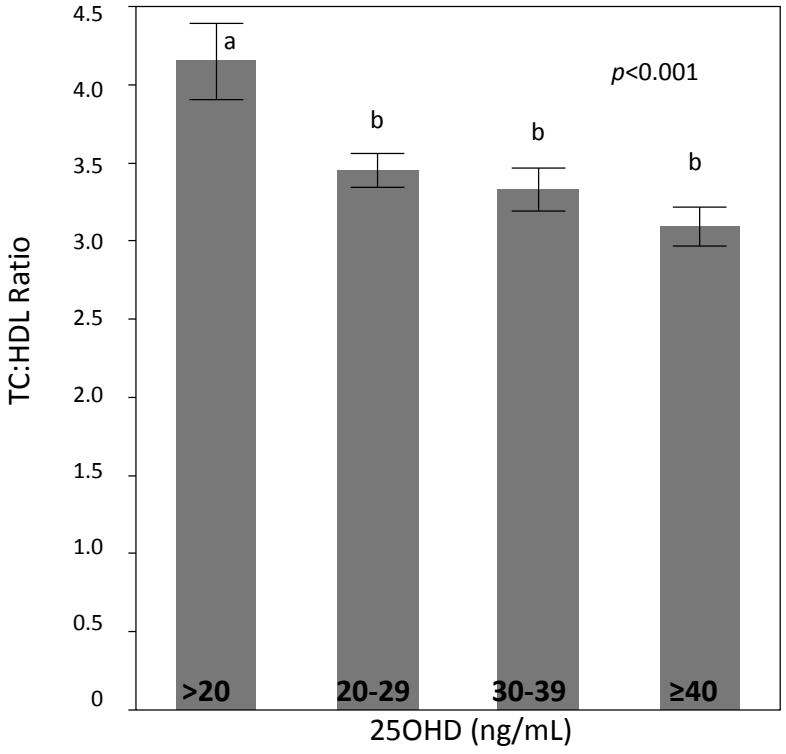


Figure 6. Mean TC:HDL ratio across vitamin D concentration ranges. Different letters indicate statistically significant differences between groups and error bars indicate ±1 standard error.



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Intake of Fortified Yogurt Drink Either with or without Extra Calcium Ameliorates Systemic Inflammatory Biomarkers, including Adipokines, in the Subjects with Type 2 Diabetes. *J. Clin. Endocrinol. Metab.* 2012;97(6):2005–2011.

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APPENDIX A

RECRUITMENT LETTER

IOWA STATE UNIVERSITY
Becoming the best.

Muscle Study
HMB & Vitamin D

Date: June 20, 2013

Dear «Title». «First_Name» «Middle_Initial». «Last_Name»,

We are currently inviting healthy women and men at least 60 years of age to participate in 12-week research study to evaluate the effect of exercise, a nutritional supplement (HMB) and Vitamin D on muscle.

There is a gradual loss of muscle and strength with age and this loss increases the risk of falls and fractures in older adults. Therefore, this research study is designed to provide effective strength and bone-health recommendations that may help reduce the risk of fractures and falls and thereby, improve the quality of life and health in older adults.

HMB (β-hydroxy-β-methylbutyrate) is the dietary supplement is being tested as part of this study. HMB comes from and amino acid (leucine). Amino acids are the building blocks of protein, which make up muscle. HMB is present in foods and is also made in our body after we eat protein (e.g. meat). Some recent research in older adults shows that HMB supplementation tends to increase muscle strength and mass with significant fat loss.

Vitamin D is a fat-soluble vitamin that can be obtained from sun exposure, food or supplements. Vitamin D has widespread effects on metabolism in the body beyond its role in calcium absorption and bone health. Vitamin D has also been associated with muscle strength, and Vitamin D supplementation in deficient individuals has been shown to improve muscle strength.

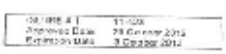
Participation criteria: At least 60 yrs of age; healthy women and men; willing to exercise 3 days per week for 1 hr/day; willing to consume a nutritional supplement; willing to provide blood and urine samples

If you fit the above criteria, please consider participating in this study. If you would like more information before you decide, please contact Hector Angus at muscle@iastate.edu or 515-294-4852 (local) or 877-578-8848 (toll free). You may also visit (www.nwrc.iastate.edu) for all the details. And if you know others that might be interested, kindly pass on our contact information.

Thank you for your time and consideration.

Yours Sincerely,

Rick L. Sharp, Ph.D.
Professor
Department of Kinesiology
Iowa State University



APPENDIX B

INFORMED CONSENT

ISU IRB # 1 11-423
Approved Date: 27 June 2012
Expiration Date: 3 October 2012

IOWA STATE UNIVERSITY
Becoming the best.

Muscle Study
HMB & Vitamin D

INFORMED CONSENT DOCUMENT

Title: Nutritional Intervention for Age-Related Muscular Function and Strength Losses

Investigators: Rick L. Sharp, PhD; John Rathmacher PhD; Marc Shulman, MD; Dr. Michael Spurlock, PhD; Joanne Lasrado, PhD; Hector Angus, MS; Jeanne Stewart, MS

This is a research study. Please take your time in deciding if you would like to participate. Please feel free to ask questions at any time.

INTRODUCTION

HMB (β-hydroxy-β-methylbutyrate) is a dietary supplement that comes from the amino acid leucine. HMB is present in foods and is also made in your body after you eat protein (meat).

Vitamin D is a fat-soluble vitamin that can be obtained from sun exposure, food or supplements. Vitamin D has widespread effects in metabolism in the body beyond its role in calcium absorption and bone health.

Therefore, the primary purpose of this research study will be to test the effect of the dietary supplement HMB with and without Vitamin D to prevent and reverse muscle wasting, and improve muscular strength and functionality in older adults. A secondary aim is to determine if HMB and Vitamin D increase lean mass and improve markers of bone turnover in adults aged 60 plus years. Bone health in terms of bone mineral content and bone mineral density will also be evaluated as secondary outcomes.

You are being invited to participate because you

- are male or female who is at least 60 yrs of age,
- are free from liver and kidney diseases,
- have no evidence of uncontrolled hypertension,
- are not morbidly obese,
- are willing to participate 3 times per week in a monitored strength-training program, and
- are willing to consume one of the nutritional supplements for the study period.

You should not participate if you

- are on high-dose Vitamin D Therapies,
- have any serious acute or chronic medical condition or illness, that would prevent you from participating in an exercise program
- have acute or chronic diseases that affect calcium or bone metabolism and health (ex. asthma with chronic use of high dose steroids, inflammatory bowel disease, Crohn's disease, primary hyperparathyroidism, seizure disorder with use of phenobarbital, etc),
- have been diagnosed with osteoporosis,
- are unable to perform exercises or if your physician has restricted exercise
- have had major surgery in the past 6 weeks,
- have had minor surgery in the past 3 weeks, or

- have evidence of uncontrolled diabetes mellitus, or Type I diabetes mellitus requiring insulin for glucose control.

DESCRIPTION OF PROCEDURES

Informed Consent and Screening (Visit 1, 60 min):

If you agree to participate, you will come in following an overnight fast (12 hr). You are encouraged to drink only water during the fast to prevent dehydration. You will read and sign the informed consent. You will be given the opportunity to ask any questions and are also free to do so at any point during the study.

You are advised to abstain from alcohol consumption for at least 48 hrs prior to the blood draw. Metabolic profile values obtained after consumption of large quantities of alcohol consumption are often erroneous and most likely present abnormal values.

Failure to carry-out the overnight fast will result in your appointment being rescheduled.

1. Your **height, weight** will be measured and your **BMI** (Body Mass Index, kg/m^2) determined.
2. Your **vital signs** (heart rate and blood pressure) will be measured**.**
3. Following completion of the measurements, there will be a **blood draw**. About 30 ml (2 tablespoons) of blood will be collected to estimate your baseline biochemical profile (including glucose, blood fat profiles, liver enzymes, Vitamin D status). In addition, your blood will be analyzed for bone specific alkaline phosphatase (BAP), c-terminal telopeptide crosslinks (CTx), 25-OH Vitamin D and PTH analysis. These measurements will give us information about your bone health.
4. You will provide a **urine sample**, which will be the second void of the morning. You may collect this at your home before you come to the laboratory. However, you will have to keep the sample refrigerated until you arrive at the laboratory for testing. You may also provide this sample at the laboratory when you arrive. A urinalysis will be conducted on your urine and will be analyzed for HMB.
5. You will be served a light **breakfast** after the blood draw.
6. You will fill out questionnaires: health, medical history and subject information.
7. Your functional mobility, balance and agility will be assessed using an **Up-&-Go Test,** which is the time it takes for you to rise from a chair, walk around a cone 8 feet in front of the chair and return to the chair.

Signed approval from your primary care provider is necessary before you can begin participation in the study. You will provide the name of your medical provider and we will contact them on your behalf. The signed approval will be sent to Dr. Rick Sharp at fax

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(515/294-8650). We will contact as soon as we have heard back from your medical provider regarding your eligibility in the study. You may also contact your doctor and obtain signed approval from them. However, we would need this approval within 5-7 business days after your screening visit or your participation may be terminated. You may send this signed approval to Dr. Rick Sharp at fax (515/294-8650), drop it off with one of the study researchers or mail it to 250 Barbara E. Forker Building, Iowa State University, Ames, IA 50011. We will contact as soon as we receive this document from you.

These preliminary tests will be used to determine your eligibility into the study. There is no compensation for the informed consent and screening visit.

Treatments, Testing and Exercise Training Schedule

If you qualify to participate, you will be randomized to the one of four treatment groups:

1. Control or placebo group,
2. HMB consuming 3.0 g/day,
3. 2000 IU Vitamin D per day,
4. 2000 IU Vitamin D + HMB, 3.0 g/day

No matter which treatment group you are assigned to, you will consume the dietary supplement **2 times per day for 12 weeks**. The supplementation will require you take a total of **6 capsules daily, 3 in the morning and 3 in the evening**. You may consume the capsules with meals.

The pills will be supplied to you in a bottle every week and you will return the pill bottle each week with the pills you have not consumed (should there be leftovers) when you come to collect the next bottle.

You will also participate in a 3-day per week exercise program for 12 weeks. Each exercise day will require about 60 minutes in the clinical laboratory (details in exercise training section).

You will complete testing at the beginning of the study (week 0), 4 weeks, 8 weeks and end of the study (week 12). Each testing session will last about 90 minutes (details in testing section).

Exercise Training (3-day/week for 12 weeks; 60 min; number of visits will depend on your proficiency with the exercise regimen)

All training will be conducted according to the guidelines of the American College of Sports Medicine (ACSM). Exercise training sessions will be supervised by research personnel who are experienced in administering the exercise tests.

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You will participate in a 3-day per week exercise training program consisting of strength training exercises utilizing Theraband® stretch cords and jumping. It is advised that you not eat anything 30 minutes before performing the exercise.

Each time you come to exercise you should either carry with you or wear the appropriate clothing and shoes. Your blood pressure and pulse rate will be measured prior to the start of the training.

You will be instructed on how to perform each exercise and each exercise session will be supervised.

The strength program will incorporate the following exercises: bicep curls, tricep extensions, chair squats, calf raises, ankle dorsiflexion, shoulder front raises and lateral raises, lat pull down, chest press, seated row, knee flexion and extension, and hip flexion.

You will complete each of the 12 exercises or movements for 15 repetitions and repeat this for two sets; a third set will be performed, but you will perform as many repetitions as you can up to 20 repetitions in good form. When you can do the third set 20 times, the resistance will be increased by moving to the next color of the resistance band.

Between each set of exercises, you will perform 5 hops or small jumps. Initially, 5 hops or jumps will be performed following each set of the 12 exercises. The number of hops/jumps will increase by five, every 3 weeks until 25 hops/jumps are achieved. You will remain at 25 hops/jumps between sets for the remainder of the study. The number of hops/jumps will be reduced or omitted if there are any complaints regarding joint pain.

Instruction on how to perform the lifts and hops/jumps will be provided by one of the project workers. Detailed instructions and demonstrations will be performed to help you learn the proper way to perform the lifts focusing on the specific muscle groups of interest. Methods to help with balance and prevent falls during the resistance exercise and the jumps will be demonstrated, but you will be supervised/assisted during the actual jumping so as to prevent falls.

Testing (4 visits; 90 min each)

These following tests will be conducted on every testing day. The testing days will be scheduled at the beginning of the study (week 0), 4 weeks, 8 weeks and end of the study (week 12) and you will be scheduled between 7 am and 9 am for testing.

You will maintain a diet record during the weeks 0, 4, 8, and 12 of testing where you will write down everything you eat and drink including water and the provided treatments for 3 days (2 weekdays and 1 weekend day). You will return the diet record to a researcher whenever you come in for testing or arrange for another time when you will be able to drop-off the diet record.

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You will come in on all testing days following an overnight fast (12 hr). You are encouraged to drink only water during the fast to prevent dehydration. Failure to carry-out the overnight fast will result in your appointment being rescheduled.

1. Your **height, weight, blood pressure,** and **heart rate** will be measured. **(Same as screening).**

2. You will provide a **blood sample during the weeks of 4, 8, and 12. (Same as screening).**

3. You will provide a **urine sample during the weeks of 4, 8, and 12. (Same as screening).**

4. Your body composition will be measured three different ways:

   a) **Dual-energy X-ray Absorptiometry (DXA)**: DXA will be used to measure changes in muscle and fat mass at only at weeks 0, 8, and 12 weeks, not week 4. DXA also provides a measure of bone density. You will lie flat on your back and the machine will slowly scan your body. You will hear the machine noise as the arm moves the length of the table. The machine will expose you to a very small amount of x-ray radiation (see comparison below) which will measure your body and bone composition. You will feel nothing during this process.

   For Females only: All female participants will be asked to complete a pregnancy risk acknowledgement prior to the DXA scan. Women with a child-bearing potential will be administered a urine pregnancy test as a safety measure to ensure that no pregnant female is exposed to X-rays. Female participants found to be pregnant will be excluded from further participation and will be advised to see their medical care provider.

   Also note that all DXA scans will be evaluated by a medical doctor who will determine whether you have osteoporosis and if it is safe for you to participate in this study.

   b) **Bioelectrical Impedance Analysis (BIA)** (same as screening)

   c) The **BODPOD** is a computerized closed-chamber in which the participant's body composition is estimated by air displacement. While the chamber is relatively small, there is a large glass window in the front and you will be in constant communication with the technician. You will be required to wear snug fitting clothing such as spandex or swimming suits while sitting inside the closed-chamber. The measurement lasts about 5-8 minutes. Hence, you will have to bring/wear under your clothes a snugly fitting swim-suit. The swim-suit should not have any wires or loose fabric since that affects the accuracy of the measurement. We have a male and female BODPOD operator. If you feel uncomfortable and would prefer a person of the same gender

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operating the BODPOD, do not hesitate to mention it to the any one of the researchers.

5. Your **leg and elbow strength** will be measured using a special machine called a isokinetic dynamometer. The machine measures the strength you are able to apply as the machine arm travels away from you (extension) and towards you (flexion).

6. Your **handgrip strength** will be measured using a handgrip dynamometer, which is a small device similar to a nutcracker which you will squeeze with your hand while the instrument records your strength.

7. Your functional mobility, balance and agility will be assessed using an **Up-&-Go Test** (same as screening).

8. You will also complete questionnaires relating to your health, how you feel, and how you perceive your health.

9. During the week 12 visit, you will complete a final questionnaire about your thoughts on the treatment you were in.

RISKS

While participating in this study you may experience the following risks:

Risks associated with exercise include muscle soreness. Severe risks include strains and sprains and possibly stress fractures. There is a potential for muscle strains to occur during strength training sessions. However, the exercise sessions will be supervised and you will be instructed and trained on the correct way to perform each exercise to minimize the chance of experiencing muscle soreness, a strain or sprain. To minimize risks all participants will perform a warm-up and cool down.

Additionally, there is the risk of falling during participation in the exercises. All reasonable care will be taken to protect against this, such as, using a chair or bar to stabilize yourself if necessary and having a trained technician act as a spotter to help you maintain balance.

It is also possible that you may experience some lightheadedness or the feeling of fainting when exercising. If you experience symptoms of being lightheaded, sit down and inform the exercise technician immediately.

There are possible, but minimal risks during blood draws such as slight discomfort, bruising, swelling, or in rare occasions, bleeding at the site of blood withdrawal. However, these risks will be minimized since blood samples will collected under strict aseptic conditions by an experienced phlebotomist.

With vitamin D supplementation there is some risk of developing vitamin D toxicity when vitamin D status becomes extremely high. Developing symptoms of toxicity are unlikely at daily intakes below 10,000 IU/day. The dose of 4000 IU per day has been set by the Institute of

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Medicine as the Tolerable Upper Limit (UL). We will assess vitamin D status throughout the study and assess your blood calcium to minimize risks.

Radiation risk from x-rays associated with the bone measurements (DXA) is minimal. Every person is exposed on a daily basis to a certain amount of background radiation originating from soil, rocks, outer space, and within the body itself. The total amount of radiation received by participating in this study through the DXA scan exposures about 4.5 mrem, which is less than what a person would receive during a transcontinental round-trip air flight (approximately 5.0 mrem) and well below the 500 mrem annual public exposure limit for infrequent exposures and the 100 mrem annual public exposure limit for frequent or continuous exposures recommended by the National Council for Radiation Protection. In Iowa, the Iowa Department of Public Health (IDPH) enforces the yearly exposure limit of 100 mrem.

BENEFITS

You will gain valuable information about your health. This includes information regarding diet, physical fitness, body composition, regional body composition and bone health status. These assessments are costly in a clinical setting and will be free to you for participating in the study. This research will provide benefits to others; once we better understand the effect of the supplementation and exercise in older adults, we will be able to provide additional effective strength and bone-health interventions for this "at risk" population.

COSTS AND COMPENSATION

You will not have any costs from participating in this study other than your time and cost of transportation to and from the testing and training site. You will be compensated $100 for participating in this study. There is no compensation for the screening visit. Your compensation will be paid to you at the conclusion of your participation (at the end of the 12 week intervention). If for any reason you are unable to continue in the study and/or choose to discontinue participation part way through the study, your compensation will be pro-rated depending on the number of weeks completed (for example, completion of 6 weeks will result in compensation of $50).

You will need to complete a form to receive payment. Please know that payments may be subject to tax withholding requirements, which vary depending upon whether you are a legal resident of the U.S. or another country. If required, taxes will be withheld from the payment you receive.

You will need to provide your social security number (SSN) and address on the form in order for us to pay you. This information allows the University to fulfill government reporting requirements. Confidentiality measures are in place to keep this information secure. You may forego receipt of payment(s) and continue in the research study if you do not wish to provide your social security number and address. Information regarding documentation required for participant compensation may be obtained from the Controller's Department; 294-2555 or http://www.controller.iastate.edu.

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PARTICIPANT RIGHTS

Your participation in this study is completely voluntary and you may refuse to participate or leave the study at any time. If you decide to not participate in the study or leave the study early, it will not result in any penalty or loss of benefits to which you are otherwise entitled.

In filling out surveys, you can skip any questions that you do not wish to answer.

You are entitled to review, and to a copy of all data collected from you at the completion of the study.

If you fail to meet all the inclusion criteria, or meet one of the exclusion criteria during the course of the study your participation will be terminated and you may be referred to your physician for further follow up.

Specifically, if during the Week 0, 8, 12 DXA, we notice abnormalities that may suggest that you have undiagnosed osteoporosis, your participation will be terminated and you will be strongly advised to see your physician immediately for a proper diagnosis, and treatment.

For your own health and safety, you are responsible for informing research personnel if there are any changes to your health, diet, lifestyle, and medications, and/or if you incur any injury, and/or if you begin participation in any other research study. These changes may result in termination of your participation if they fulfill the exclusion criteria, if it is unsafe for you to continue participating or if these changes interfere with the study objectives.

You will not be permitted to participate if we are unable to obtain approval for your participation from your physician.

Also, if during the course of the study you develop a medical condition or injury that would not automatically exclude you from participating you may be required to provide approval from your Primary Care Physician, confirming that it is still safe for you to continue participating in this study.

During the course of the study Vitamin D or calcium levels in your blood will be monitored and if Vitamin D values appear to be above normal values, your participation will be terminated immediately and you will be advised to see your physician.

If compliance is deemed insufficient (e.g. supplement not being consumed, diet records incomplete, inadequate performance during exercise training) to the principal investigator your participation may be terminated.

Failure to show up to 3 consecutive appointments may result in termination of participation.

Failure to carry-out an overnight fast on 3 consecutive occasions may result in termination of participation.

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RESEARCH INJURY

Emergency treatment of any injuries that may occur as a direct result of participation in this research is available at the Iowa State University Thomas B. Thielen Student Health Center, and/or referred to Mary Greeley Medical Center or another physician or medical facility at the location of the research activity. Compensation for any injuries will be paid if it is determined under the Iowa Tort Claims Act, Chapter 669 Iowa Code. Claims for compensation should be submitted on approved forms to the State Appeals Board and are available from the Iowa State University Office of Risk Management and Insurance.

CONFIDENTIALITY

Records identifying participants will be kept confidential to the extent permitted by applicable laws and regulations and will not be made publicly available. However, federal government regulatory agencies, Food and Drug Administration, National Institutes of Health, National Institute of Aging, Office of Human Research Protection, auditing departments of Iowa State University, and the Institutional Review Board (a committee that reviews and approves human subject research studies) may inspect and/or copy your records for quality assurance and data analysis. These records may contain private information. This study is also being conducted under an Investigational New Drug application and as such the Food and Drug Administration may inspect or copy records.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

To ensure confidentiality to the extent permitted by law, the following measures will be taken:

- The data collected from the study will be regarded as privileged and confidential.
- You will be assigned a unique identifier code and all the information you provide will be listed under your code.
- There will be only one hard copy with your name/identity and all information (e.g. questionnaires, diet record, clinical measures) will be stored in a secure filing cabinet. This cabinet can only be accessed by the PI and co-investigators.
- There will only be one file maintained on a password protected server, one back-up file on a CD/USB and one hard-copy connecting your name with this unique identifier code. This file can only be accessed by the PI and co-investigators.
- The de-identified data will be kept indefinitely and if the results are published or presented, your identity will remain confidential.
- Your data will be shared with researchers in South Dakota State University, but researchers there will not have access to your identity, which will remain confidential.

- Should it become necessary or desirable to release identifiable health information, a disclosure authorization will be obtained from you prior to release of the information. A record of all disclosures and authorizations will be kept with your information. All records and information will be stored for a minimum of 3 years past the last subject finishing study protocols.

- The sponsoring company (Metabolic Technologies, Inc, Ames, IA) will receive the data files from the trials, but will not have access to your identity, which will remain confidential.

QUESTIONS OR PROBLEMS

You are encouraged to ask questions at any time during this study.

- For further information about the study contact Dr. Rick Sharp (515) 294-8650 or Hector Angus (515) 294-8481.

- If you have any questions about the rights of research subjects or research-related injury, please contact the IRB Administrator, (515) 294-4566, IRB@iastate.edu, or Director, (515) 294-3115, Office for Responsible Research, Iowa State University, Ames, Iowa 50011.

****

PARTICIPANT SIGNATURE

Your signature indicates that you voluntarily agree to participate in this study, that the study has been explained to you, that you have been given the time to read the document, and that your questions have been satisfactorily answered. You will receive a copy of the written informed consent prior to your participation in the study.

Participant's Name (printed) _____

_____ _____
(Participant's Signature) (Date)

_____ _____
(Researcher Signature) (Date)